Insecticide Resistance Enzyme Activity Protocol

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1: Sample Preparation for Microplate Assays

Adapted from: Methods in Anopheles Research Manual section 5.2 “Microplate Enzyme Activity Assays.”[1] The following changes were made to that protocol to improve accuracy and clarity and interpretation of results (noted below with an *). Specifically, the previous version did not consider the importance of body size, age and mosquito body condition. We added details to ensure that mosquitoes are reared from eggs under standard diet and conditions to a uniform age. This is important because body size, age and body condition can alter influence enzyme expression results. In addition, we found that mosquitoes previously exposed to pesticides (taken from bottle or other assays) are not suitable for determining baseline enzyme levels, as pesticide pre-exposure can reduce enzyme activity. We found that it was important to add a centrifuge step prior to making aliquots of each sample to remove excess chitin and other debris. We also recommend storage of initial mosquitoes and processed samples at -80°C to ensure optimal sample preservation. We also recommend that samples should be used within 7 days of processing, as we determined that enzyme degradation occurs over time once processed. Finally, we provide instructions for data analysis with R package platr as well as interpretation of the results.[3,4,5]

General Materials

- Costar 3795 microplates with 330 µL round bottom wells
- Microplate reader
- vortexer
- Analytical balance
- Fume hood
- Graduated cylinders, 25 mL, 100 mL and 500 mL
- pH meter
- Laboratory magnetic stir bar and plate
- Acid resistant pipet tips, 200 µL and 1000 µL
- Microcentrifuge tubes 1.5 mL
- Storage bottles 1 L

- Multichannel pipettes 20-200 µL and 10-50 µL

Sample Preparation Materials

- Dibasic potassium phosphate (Vol = 6.6 g) Fisher 447362500
- Monobasic potassium phosphate (Vol = 1.7 g) Fisher 424200250
- Handheld motorized pestle

Reagent Preparation:

- 0.1 M Potassium phosphate buffer (KPO_4)
  1. Place 800 mL purified water (Milli Q) in a glass beaker on a stirrer
  2. Add 6.6 g anhydrous dibasic potassium phosphate
  3. Add 1.7 g monobasic potassium phosphate
  4. Adjust to pH 7.2 using one of the above.
  5. Adjust to 1000 mL final volume
  6. Store at room temperature (discard if potassium has precipitated out of solution)

Protocol for mosquito preparation:

Important: Sample preparation must be performed on ice as the enzymes will degrade rapidly at room temperature.

1. Remove mosquitoes from -80°C freezer.
   a. Use 3–5-day old female mosquitoes**
   b. Mosquitoes should be reared from eggs to obtain consistent size and nutrition**
   c. Do not use mosquitoes that have been exposed to pesticides already (e.g., used in bottle bioassays, etc.).**
2. Label two sets of tubes; one set will contain the mosquito body, which should be indicated on the label.
   a. Sample tube side: Species, Year extracted, Submission/sample ID
   b. Sample tube top: Submission/Sample ID, Sample Tube number
3. On ice, homogenize each sample or pool in 100 µL of KPO_4 buffer in a1.5 mL microcentrifuge tube, grinding each sample with a clean pestle for approximately 20 seconds.
4. After grinding, dilute samples with 900 µL of KPO_4 to a total final volume of 1000 µL.
5. Centrifuge for 3 min at 25000 RCF, or g force, to move solids to the bottom of the tube. **
6. Aliquot 500 µL of the supernatant into the second batch of labeled 1.5 mL microcentrifuge tubes. Do not disturb the pellet at the bottom of the tube. Do this step immediately after step 5.
7. Dilute both sets of tubes with 500 µL of KPO_4 to a final volume of 1000 µL in each tube.
8. Store processed samples in a -80°C freezer for up to 7 days. Process and handle susceptible controls in the same manner as sample mosquitoes throughout the entire process.**

2: Nonspecific Esterase Assay

Adapted from: Methods in Anopheles Research Manual section 5.2 "Microplate Enzyme Activity Assays."[1] We added chemical handling guidance and recommendations for personal protection due to the corrosive nature of reagents used in the assay. Reagent storage parameters were added or adjusted

from the original protocol to meet manufacturer recommendations for optimal storage. For example, naphthyl acetate solution should be made on the same day of the assay and the dianisidine solution should be made no more than one hour prior to reading sample plates. We found that the esterase activity stock solution (positive control) can be made ahead of time and stored in aliquots in a -80°C freezer. We tested stock aliquots one year after storage and found no evidence of degradation, therefore we recommend storage up to 1 yr. We expanded our dilution series from the original protocol 1:55 and a 1:110 dilution to a 9-part serial dilution series. This series is simpler to perform (minimizing error) and covers the full range of absorbance in the linear portion of the absorbance standard curve. We also adjusted the sample and reagent volumes to accommodate the well size of our microplates.

Materials

- Acetone
- MilliQ water
- 0.25 M KPO_4 (Storage: Room temperature)
    - From sample preparation protocol
- α or β-naphthyl acetate (Sigma, α N8505 or β N6875) (Storage: -20**°C**)
- 0-dianisidine tetrazotized (Sigma, D9805-10G) (Storage: 4**°**C)
- α or β -naphthol (Sigma, α N1000-10G or β 185507-5G) (Storage: -20**°C**)
- Brown glass storage bottles (100 mL)

Note: This assay involves several toxic or corrosive chemicals that require personal protection equipment (PPE; laboratory coat, close toed shoes, gloves and protective eye wear). These items should be used when handling chemicals and preparing samples for plate preparation. All sample and reagent preparation should be conducted in a chemical laboratory hood. In addition, all equipment susceptible to corrosion should be thoroughly wiped with acetone after use.

Reagent Preparation

Esterase activity stock solution (Positive Control) (Full PPE, chemical hood)

1. Dissolve 50 mg α or β-naphthol in 10 mL acetone, depending on the assay.
2. Add 90 mL of 0.25 M KPO_4
3. Place 1 to 1.5 mL aliquots of solution in microcentrifuge tubes and freeze. Use a lightproof storage container.
    - Stock solution can be stored at -80**°**C; solution tested approximately one year after storage showed minimal degradation.**

Dilution Series

1. Create the 1:2 serial dilution using the originally prepared stock solution.
    a. The dilution series outlined in Table 1 will create enough solution in each vial for three assays of 120 µL each.**

Table 1: Steps for creating dilution series for the esterase positive control.

	Esterase Serial Dilution Series									
Sample #	1	2	3	4	5	6	7	8	9	10
Stock conc ug/ul	0.5	0.25	0.125	0.0625	0.03125	0.01562	0.007813	0.00390	0.001953	0.00097
Dilution Factor	2	2	2	2	2	2	2	2	2	2
(+) Ctrl (Previous tube)	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
KPO_4	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
Final Vol	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	1000 µL
Final Conc ug/ul	0.25	0.125	0.0625	0.03125	0.01562	0.007813	0.00390	0.001953	0.00097	0.00048

α or β-naphthyl acetate (Full PPE)

1. Dissolve 56 mg α or β-naphthyl acetate in 20 mL acetone.
2. Add 80 mL 0.25 M KPO_4.
3. It is important to prepare fresh solution each day that assays are conducted. Solution should be prepared at room temperature.

Dianisidine solution (Fast Blue Salt)

1. Dissolve 100 mg 0-dianisidine tetrazotized in 100 mL purified water no more than one hour before use.**
2. Store a light-proof bottle until ready for use. Check the color of dianisidine before use. The color should be pale yellow. If the color is amber, discard and make a new batch.

Microplate Assay

1. Remove samples from the freezer approximately one hour before use and thaw completely on the bench top at room temperature.
2. Briefly, vortex samples.
3. Load 60 µL of the mosquito homogenates into each well of a microplate (Costar 9795). Starting with well B1 load each sample in duplicate (Table 2), moving from left to right across the plate. Sample 1 will be in wells B1 and B2. Each plate will hold 36 samples.
4. Place samples back in the -80°C after loading.
5. Load 60 µL of KPO_4 to the negative control wells A11, A12, H1 and H2.
6. Positive Control = α or β –naphthol. Add 60 µL of the appropriate dilution to each plate as a positive control. A1- A10 and H3 - H12.

Table 2: Plate map showing how to load samples, positive and negative controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**(-)**	**(-)**
B	1	1	2	2	3	3	4	4	5	5	6	6
C	7	7	8	8	9	9	10	10	11	11	12	12
D	13	13	14	14	15	15	16	16	17	17	18	18
E	19	19	20	20	21	21	22	22	23	23	24	24
F	25	25	26	26	27	27	28	28	29	29	30	30
G	31	31	32	32	33	33	34	34	35	35	36	36
H	**(-)**	**(-)**	**D10**	**D9**	**D8**	**D7**	**D6**	**D5**	**D4**	**D3**	**D2**	**D1**

7. Add 60 µL α or β-naphthyl acetate to each well using a multichannel pipette moving from column 1 to column 12.
8. Start a 1 min timer after the last column of reagent is added. During that 1 min, place the microplate into the plate reader (Biotek 800TS). Start the protocol when 1 min is over. The machine plate reader will shake the plate for 9 min for a total incubation time of 10 min.
9. After the 9-minute shake, the plate will be ejected from the machine.
10. Remove the plate and quickly add 60 µL dianisidine to each well using a multichannel pipette and then return the plate to the plate reader. It is important to work quickly in the next step. The plate reader protocol allows 90 secs to load each well with the dianisidine solution.
11. Return the plate to the microplate reader and incubate for 2 min.
12. After 2 mins, the plate reader will automatically read sample absorbance at 630 nm if using α-naphthyl or at 540 nm if using β-naphthyl acetate.
13. Save the data in the correct project folder, (ie. Sub2023 for submission samples from 2023) with a consistent file name that corresponds to the project. You will want the file name to contain enough information to track samples. For example, a file for Sub2023 is Sub2023_Beta_Pip_Col_567_5001_1128. This is a beta esterase assay with *Cx. pipiens* for colony controls and two submissions, 567 and 5001 and it was run on the 28th of Nov.
14. Convert it to an Excel document after saving the original file. Upload to a box folder for backup.

3: Microplate Oxidase Assay

Adapted from: Methods in Anopheles Research Manual section 5.2 “Microplate Enzyme Activity Assays.”[1] We made several change to the Brogdon 2015 protocol to improve accuracy. Reagent storage parameters were added or adjusted from the original protocol. For example, the TMBZ and hydrogen peroxide solutions should be made fresh on the same day of the assay, the cytochrome-C positive control can be made ahead and stored as aliquots in a -80°C freezer. Cytochrome-C must be made with sodium acetate buffer rather than potassium phosphate (correcting an error in the Brogdon 2015 protocol); we added additional guidance because cytochrome-C is difficult to make. As with the nonspecific esterase assays (above), we expanded our dilution series from the original protocol 1:55 and a 1:110 dilution to a 9-part serial dilution series to cover the full range of absorbance in the linear portion of the absorbance standard curve. We also adjusted the sample and reagent volumes to accommodate our microplates.

Materials

- Pure methanol - Fisher A452-1
- Sodium acetate - Fisher S93352
- Glacial acetic acid - Fisher A38^{c}-212
- Purified water
- TMBZ or TMBZ[2HCl] - MP Biomedicals, LLC 980502
- >3% hydrogen peroxide (H_2O_2) - Fisher H325-500
- 0.25 M KPO_4
- Cytochrome-C from bovine heart - Sigma c3131
- Brown glass storage bottles (100 mL)

Reagent preparation

0.25 M Sodium acetate trihydride Buffer [NaOAc] (For use in TMBZ solution). *If using anhydrous sodium acetate (see below).

1. Place 900 mL purified water in a glass beaker on a stirrer.
2. Add 83 mL 3M sodium acetate.
3. Adjust to pH 5 with glacial acetic acid.
4. Adjust to 1000 mL final volume.
5. Store at room temperature.
6. Note: 3M NaOAc can be purchased or made by dissolving 408. 1g of NaOAc in 800 mL of water. Once dissolved, adjust the final volume to 1 L.

*Note: If using anhydrous sodium acetate, the MW is 82.03 g, so for a 3 M solution, you would use 246.09 g added to enough water (approximately 900 mL) to reach 1000 mL.

TMBZ solution

1. Dissolve 20 mg 3,3',5,5'-Tetramethyl-benzidine dihydrochloride* (TMBZ [2HCL] or TMBZ) in 25 mL methanol. TMBZ will dissolve if left on the benchtop for a few minutes or swirled under hot water from the tap. Do not shake or heat with an open flame or on a hot pad as the solution is flammable.
2. Add 75 mL 0.25 M Na acetate, pH 5.0 buffer (prepared above).
    a. If completing only one plate: 4 mg of TMBZ into 5 mL methanol. Add 15 mL of 0.25 M NA acetate. This makes approximately 20 mL of solution.
3. TMBZ solution should be made fresh each day, stored TMBZ will lose reactivity over time.**

3% hydrogen peroxide

1. Hydrogen peroxide is available in many concentrations. Prepare a 3% solution in purified water. Make this fresh for each day to ensure reactivity.**

Oxidase positive control stock

1. Add 10 mg Cytochrome-C to 100 mL 0.25 M Na acetate buffer [NaOAc], pH 5.

a. For a partial stock, weigh out as close to 1 mg as possible, adjust sodium acetate volume to the amount weighed.
b. Weigh carefully. Turn off machines close to scale to prevent airflow and vibration. Tare a 50 mL beaker and measure the powder into it. Cover with Kim wipe when moving the sample from the scale to the bench.
    i. Cytochrome-C is fluffy and sticky; the powder will blow out of the beaker if you are not careful.
c. Add sodium acetate to the beaker, gently swirl to mix and pour into a 25 mL dram. Cover immediately with foil.
d. Store in -80°C in aliquots as needed. 250 µl of control is needed for each assay.**
    i. Note: This control will lose stability over time, long-term storage for liquid aliquots is not reliable; use within a month.**

Dilution series

2. Create the 1:10 dilution using the originally prepared stock solution (see bold samples below). Initial dilutions (1 and 1.5) will be used to create a 9-fold 0.011 dilution series (2-9) in Table 1. Tubes **1.1** and **1** are used to create **all following dilutions.**
    a. The dilution series outlined in Table 1 will create enough solution in each vial for three assays of 120 µL each.
    b. It is essential that sodium acetate is used to create dilutions. KPO_4 will lead to rapid degradation and inaccurate representations of the activity of the samples.**

Table 1: Steps for creating a serial dilution series for the oxidase positive control.

	Oxidase Dilution Series									
Sample #	1	2	3	4	5	6	7	8	9	10
Stock conc ug/ul	0.1	0.05	0.025	0.0125	0.00625	0.003125	0.001562	0.000781	0.00039	0.000195
Dilution Factor	2	2	2	2	2	2	2	2	2	2
(+) Ctrl (Previous tube)	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
KPO_4	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
Final Vol	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL	1000 µL
Final Conc ug/ul	0.05	0.025	0.0125	0.00625	0.003125	0.001562	0.000781	0.00039	0.000195	0.000097

Microplate assay

1. Remove samples from the -80°C freezer approximately 1 hr before use and thaw completely on the bench top at room temperature.
2. Briefly, vortex samples.
3. Add 60 µl of mosquito homogenates into each well of a microplate (Costar 9795). Starting with well B1 load each sample in duplicate (Table 2), moving from left to right across the plate.
4. Place samples back in the -80°C freezer loading.

5. Load 60 µl of KPO4 to the negative control wells A11, A12 and H1, and H2.

Table 2: Plate map showing how to load samples, positive and negative controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**(-)**	**(-)**
B	1	1	2	2	3	3	4	4	5	5	6	6
C	7	7	8	8	9	9	10	10	11	11	12	12
D	13	13	14	14	15	15	16	16	17	17	18	18
E	19	19	20	20	21	21	22	22	23	23	24	24
F	25	25	26	26	27	27	28	28	29	29	30	30
G	31	31	32	32	33	33	34	34	35	35	36	36
H	**(-)**	**(-)**	**D10**	**D9**	**D8**	**D7**	**D6**	**D5**	**D4**	**D3**	**D2**	**D1**

6. Positive control = cytochrome-C. Load 60 µl of the appropriate dilution to each plate as a positive control. A1 – A10 and H3 – H12.
7. Add 120 µl TMBZ solution to each well using a multichannel pipette, moving from column 1 to column 12.
8. Add 15 µl 3% hydrogen peroxide (H_2O_2) to each well using a channel pipette moving quickly across the plate (reaction begins here).
9. Start a timer for 1 min as soon as the H_2O_2 is added to the final column. Place the plate into a microplate reader (Biotek 800TS). Start the protocol after the 1 min is over. The plate will be shaken in the reader for an additional 4 min to ensure mixing, stabilize the meniscus, and reduce air bubbles.** Total incubation time for this assay is 5 min.
10. Read the plate after 4 minutes at 620 - 630 nm (already programmed in the plate reader).

4: Analysis

1. Export data from the microplate reader into an Excel format and save it to a project folder.
2. The R package platr is used to organize the data pulled from the microplate reader into a project wide data frame.[2] Platr allows for each well to be labeled with as many descriptors and variables as needed.**
3. The program automatically adjusts data based on the value of the negative controls to remove background noise.
4. The mean of each set of duplicate samples is then calculated.
5. Next, the proportion of field collected mosquito samples that have a higher absorbance than the 99th percentile of the susceptible samples is determined with three potential outcomes.[3,4,5]
    a. Unelevated: This means that less than 15% of the mosquito samples have enzyme activity that exceeds the 99th percentile threshold. It suggests that enzyme levels in these mosquitoes are not significantly different from those in the susceptible mosquitoes.

   b. Emerging elevation: More than 15% of the mosquito samples have absorbances above the 99th percentile threshold. This indicates that the mosquito samples are likely developing elevated enzyme levels.
   c. Elevated: This category indicates that more than 50% of the mosquito samples fall above the 99th percentile threshold. It suggests that enzyme production in these mosquitoes is elevated compared to the susceptible mosquito samples.
6. Visualize data in the form of a boxplot with data points jittered so they are all visible. Make sure that the susceptible colony mosquitoes are represented on the figure.

Tips for success

1. Rear and store susceptible mosquitoes at -80°C on a weekly or bi-weekly basis to run alongside field collected samples.
2. Do not process samples in buffer until you are ready to run microplates. Plan to run all assays within a week of processing. **
3. Create fields in the platr template for everything possible that could change between assays. This includes but is not limited to, collection location and date, date of assay, lot number of reagents, date controls were made; etc. The benefit of the platr R package is that all fields will become columns and can be tested and filtered. This is useful for troubleshooting if you get unexpected results that you suspect are erroneous.
4. Create a single page protocol to have at the bench that includes only the steps needed for that assay with a plate map and fields that will eventually be translated into the platr document.

5: Citations

1. Brogdon W, 2015. Methods in Anopheles Research: 2015 Edition. BEI Resources, Section 5.2: 248-259. https://www.beiresources.org/Portals/2/VectorResources/2016%20Methods%20in%20Anopheles%20Research%20full%20manual.pdf
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5. Polson K, Brogdon W, Rawlins S, Chadee D, 2011. Characteristics of insecticide resistance in Trinidadian strains of Aedes aegypti mosquitoes. Acta Tropica, 117(1), 31-38. https://doi.org/10.1016/j.actatropica.2010.09.005