Global Manual for Evaluating Insecticide Resistance Using the CDC Bottle Bioassay

U.S. Department of Health and Human Services
Centers for Disease Control and Prevention

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Preface

This manual is an update of the 2011 document entitled, 'Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay.'

Insecticide resistance in a mosquito population is initially detected and characterized by using bioassays to assess whether a particular insecticide is able to kill a given mosquito population. Ideally, this fundamental question should be answered before insecticide-based products are chosen and procured for mosquito control.

The U.S. Centers for Disease Control and Prevention (CDC) bottle bioassay is a surveillance tool for detecting resistance to insecticides in mosquito populations. It is designed to help determine if a particular insecticide is able to kill a vector population at a specific location at a given time. This information, combined with results of bioassays using synergists and those of biochemical and molecular assays, can assist in determining which insecticides are most likely to yield the greatest impact.

The aim of this document is to provide a practical laboratory manual that describes how to perform and interpret the CDC bottle bioassay. Further information for resistance testing, including instructional videos and a downloadable version of this manual (including versions in languages other than English), can be obtained from the CDC website at https://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html

We hope you find this tool useful in the support of vector control programs.

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We wish to thank the enormous number of people who have contributed to the development and implementation of this method, including the helpful feedback received on the 2011 document, 'Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay'. Scientists and control program personnel from many countries have expended great energy and resources in the collection of field data that have enabled the refinement and evaluation of the CDC bottle bioassay method alongside other methods, such as the World Health Organization (WHO) paper-based bioassay. While too numerous to mention individually, these people have our humble and sincere gratitude.

1. Introduction

Bioassays allow for the detection and characterization of insecticide resistance in a mosquito population. This manual describes the procedures for the U.S. Centers for Disease Control and Prevention (CDC) bottle bioassay, a tool for detecting resistance to insecticides. The information generated by this method, combined with results of bioassays using synergists and those of biochemical and molecular assays, can provide important information regarding the frequency of resistance in a population and the mechanisms associated with resistance. Taken together, vector control programs can use these data to inform their mosquito control strategies.

The CDC bottle bioassay relies on time-mortality data, which measures the time it takes for an insecticide to penetrate a vector, traverse its intervening tissues, get to its target site, and act on that site. Anything that prevents or delays the compound from achieving its objective — killing insects — contributes to resistance. Information derived from the CDC bottle bioassay may provide initial evidence that a mosquito population is developing resistance to an insecticide.

A major advantage of this bioassay method is that different concentrations of an insecticide can easily be evaluated. Furthermore, the technique is simple, rapid, and economical. The CDC bottle bioassay can be used as part of a broader insecticide resistance monitoring program, which may also include World Health Organization (WHO) paper-based bioassays, and biochemical and molecular methods.

The CDC bottle bioassay can be adapted for any insect species. For the purposes of this manual, mosquitoes will be used as the example.

2. Materials and reagents

2.1. Materials

- 250 ml Wheaton bottles with screw lids (Figure 1). Each bioassay typically requires five bottles: four for insecticide replicates and one for an untreated control
- Graduated disposable plastic pipettes that can measure 1 ml, or micropipettes and tips
- Aspirator apparatus for collecting mosquitoes
- Containers for transferring/holding mosquitoes
- Bottles for stock solutions. These can be amber-colored, or foil-wrapped if clear bottles are used (100–1,000 ml depending on the user's choice of stock solution volume)
- Timers capable of counting seconds
- Permanent markers for labeling bottles, caps, and pipettes
- Masking tape for labeling bottles, caps, and pipettes
- Lab coat and disposable gloves
- Data sheets, pens, and pencils for data recording

2.2. Reagents

- Insecticide(s) to be tested (technical grade)
- Acetone or technical grade absolute ethanol

2.3. Biological material

- Mosquitoes for testing

Note: Use safety procedures and personal protective equipment (PPE) as recommended by your institution when handling insecticides (e.g., gloves, laboratory coat).

Figure 1: Example of materials and reagents for the CDC bottle bioassay. *Accessible explanation on page 28.*

3. Initial considerations

3.1. Diagnostic dose and diagnostic time

The diagnostic dose is the dose of insecticide that kills 100% of susceptible mosquitoes within a given amount of time. The expected time for the insecticide to achieve this objective is called the diagnostic time. These are the reference points against which all other results are compared. Resistance is assumed to be present if a significant portion of the test population survives exposure to the diagnostic dose at the diagnostic time.

Ideally, the diagnostic dose and the diagnostic time should be defined for each insecticide, each region, and each mosquito species that is monitored. The diagnostic dose and the diagnostic time are validated using susceptible populations of mosquitoes. Consistent use of the same parameters is required to detect changes in the response of the population over time (e.g., the proportion of tested mosquitoes surviving after the diagnostic time that originally killed 100% of the test population). Detailed information about diagnostic doses, diagnostic times, and calibration of the CDC bottle bioassay is given in Appendix 2. Bioassay guidelines for additional insecticides are available from WHO: https://www.who.int/teams/global-malaria-programme/prevention/vector-control/insecticide-resistance.

Table 1 shows diagnostic doses and diagnostic times applicable to *Anopheles* and *Aedes* mosquito populations. The diagnostic doses and the diagnostic times for anophelines were originally agreed upon for use on anophelines collected in South America as part of the Amazon Malaria Initiative (AMI). These doses and times, as well as those listed for Aedes, are well within the range of diagnostic doses and diagnostic times for use worldwide and have been applied successfully in many countries around the world. Therefore, the diagnostic doses and the diagnostic times in Table 1 serve as sample reference points for some of the main insecticides used globally. It is important to highlight that the diagnostic doses are lower than the doses of insecticide that are typically delivered in commercial vector control products. The purpose of screening mosquito populations using diagnostic doses is to allow for the detection of resistance at an early stage of development in a mosquito population before the resistance has become sufficiently intense to hinder the efficacy of the products applied in the field. As such, bioassays can be considered an early warning tool to detect resistance before it compromises vector control operations. When bioassays are routinely used to monitor insecticide susceptibility, any change in susceptibility status will signal the need to implement interventions using alternative insecticides in order to maintain high vector control efficacy and avoid further selection for resistance.

If a diagnostic dose and diagnostic time have not yet been determined for a given species or insecticide, a calibration can be conducted to define these parameters. Detailed instructions on how to do this are presented in Appendix 2. Bioassay guidelines for additional compounds are available from WHO: https://www.who.int/teams/global-malaria-programme/prevention/vector-control/insecticide-resistance.

Table 1: Diagnostic doses and diagnostic times for *Anopheles* and *Aedes* mosquitoes

Pyrethroid insecticides

Insecticide	*Anopheles* Insecticide concentration (µg/bottle)	*Aedes* Insecticide concentration (µg/bottle)	Diagnostic time (minutes)
Permethrin	21.5	15	30
Alpha-cypermethrin	12.5	10	30
Cypermethrin	12.5	10	30
Deltamethrin	12.5	10	30
Lambda-cyhalothrin	12.5	10	30
Cyfluthrin	12.5	10	30

Carbamate insecticides

Insecticide	*Anopheles* Insecticide concentration (µg/bottle)	*Aedes* Insecticide concentration (µg/bottle)	Diagnostic time (minutes)
Propoxur	12.5	12.5	30
Bendiocarb	12.5	12.5	30

Organophosphate insecticides

Insecticide	*Anopheles* Insecticide concentration (µg/bottle)	*Aedes* Insecticide concentration (µg/bottle)	Diagnostic time (minutes)
Malathion	50	50	30
Fenitrothion	50	50	30
Pirimiphos-methyl	20	25	30

Organochlorine insecticides

Insecticide	*Anopheles* Insecticide concentration (µg/bottle)	*Aedes* Insecticide concentration (µg/bottle)	Diagnostic time (minutes)
DDT	100	75	45

3.2. Preparation of stock solutions

The bottles used for the bioassay need to be coated inside with the diagnostic dose of the insecticide under evaluation. As shown in Table 1, the diagnostic doses are specific to each insecticide and can differ between genera. Stock solutions can be prepared such that 1 ml of the stock solution would contain the desired amount of insecticide to be added to the bottle. For example, if 12.5 µg of deltamethrin is the diagnostic dose to be added to a test bottle, it would be advisable to have a stock solution containing 12.5 µg/ml. It is practical to make stock solutions with concentrations that can be easily correlated to the dose needed to coat the bottles.

To make insecticide stock solutions, dilute the appropriate amount of technical grade insecticide in technical grade acetone or technical grade absolute ethanol. Examples of quantities of technical grade insecticide needed to prepare 100 ml, 500 ml, and 1,000 ml of stock solutions are shown in Table 2. Technical grade insecticide may be solid or liquid and should not be expired.

Box 1: Weighing Powdered Insecticides Safely

- It is important to wear the appropriate personal protective equipment (PPE) per institutional policy, which typically includes gloves, safety glasses, and lab coats.
- If possible, work inside a fume hood that has a balance close by; place a sterile drape on the work surface inside the fume hood.
- Tare the balance.
- Place a standard volumetric flask (without the stopper) on the balance and tare.
- With the flask inside the hood, carefully remove the cap of the technical grade insecticide bottle and place the cap with its external side down on the drape inside the fume hood.
- Add the insecticide from the bottle to the flask with a disposable spatula while inside the hood. Carefully transfer the flask with the insecticide back to the balance.
- Weigh the insecticide and record the weight. Repeat these steps until achieving the desired weight.
- **Note:** The powdered insecticides should only be handled in the hood. The lid of the vial should remain closed between adding and removing insecticides.
- Remove the flask from the balance and return to the fume hood to add the appropriate volume of solvent (acetone or ethanol) to make the desired concentration of stock solution.
- Stopper the flask tightly to ensure that there is no loss of solvent due to evaporation. Discard the sterile drape in a biohazard waste bin and clear the workspace.
- In case of a spill, the drape needs to be placed in a plastic bag and discarded as hazardous waste. Close the cap of the technical grade insecticide bottle tightly. If the bottle is empty, place it in the designated area for hazardous waste pick-up.

It is important to label the stock solution bottle with the name of the insecticide, concentration, and date of preparation. Once the stock solution is made, it can be stored in the refrigerator (4°C) in light-proof bottles (amber-colored bottles or foil-wrapped if clear) for future use. Stock solutions of DDT and organophosphates should be used within 24 hours. At the CDC, refrigerated stock solutions of pyrethroids and carbamates have been used for 2–3 years without degradation of activity. It is recommended to take the stock solutions out of the refrigerator at least 1 hour before coating bottles to allow them to come to room temperature before use. The stock solution should be gently swirled before use in order to mix it.

Table 2: Quantities of technical grade insecticide required for preparation of different volumes of stock solution

For *Anopheles*:

Weight (mg) of technical grade insecticide needed per volume of stock solution

Pyrethroid insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
Permethrin	2.15	10.75	21.5
Alpha-cypermethrin	1.25	6.25	12.5
Cypermethrin	1.25	6.25	12.5
Deltamethrin	1.25	6.25	12.5
Lambda-cyhalothrin	1.25	6.25	12.5
Cyfluthrin	1.25	6.25	12.5

Carbamate insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
Propoxur	1.25	6.25	12.5
Bendiocarb	1.25	6.25	12.5

Organophosphate insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
Malathion	5	25	50
Fenitrothion	5	25	50
Pirimiphos-methyl	2	10	20

Organochlorine insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
DDT	10	50	100

For *Aedes*:

Weight (mg) of technical grade insecticide needed per volume of stock solution

Pyrethroid insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
Permethrin	1.5	7.5	15
Alpha-cypermethrin	1	5	10
Cypermethrin	1	5	10
Deltamethrin	1	5	10
Lambda-cyhalothrin	1	5	10
Cyfluthrin	1	5	10

Carbamate insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
Propoxur	1.25	6.25	12.5
Bendiocarb	1.25	6.25	12.5

Organophosphate insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
Malathion	5	25	50
Fenitrothion	5	25	50
Pirimiphos-methyl	2.5	12.5	25

Organochlorine class insecticides			
Insecticide	**100 ml**	**500 ml**	**1,000 ml**
DDT	7.5	37.5	75

3.3. Mosquito handling

Female mosquitoes to be used in the bioassay can be collected as adults from the field (of mixed age and physiological status) or as adults of a known age reared from field-collected larvae or eggs. Ideally, non-bloodfed female mosquitoes aged 2-5 days should be used in the bioassay. Field-collected adults can be fed and allowed to oviposit in the lab and the resulting F1 progeny used for the bioassay. It is not recommended to bioassay beyond the F2 generation, as the characteristics of wild populations can be rapidly lost upon colonization. Although not normally recommended due to the variations in susceptibility that can occur with age and other physiological characteristics, bioassays can be conducted directly on adult female mosquitoes collected from the field, with their physiological status (i.e., unfed, blood fed, semi-gravid, gravid) recorded on the result sheet. Female mosquitoes should be fed only with 10% sugar solution the day before testing. It is recommended that a minimum of 100 mosquitoes, divided among four replicate bottles, should be tested per insecticide for a given population. When it is not possible to obtain this number of mosquitoes on a single occasion, results of multiple bioassays over a few days may be pooled to achieve the recommended sample size of 100 mosquitoes. Each bioassay should include an untreated control bottle with 10–25 mosquitoes.

Some field collections may contain multiple mosquito species. If species cannot be separated prior to the bioassay, they can be sorted afterwards (Box 2).

Box 2: Guidelines for situations where multiple mosquito species exist in sample collections.

In situations where multiple mosquito species are collected, it is recommended that each species be bioassayed separately. If species are not morphologically distinguishable, molecular methods can be used to confirm species after the bioassay, taking care to ensure that the susceptibility status of each mosquito is recorded prior to conducting molecular testing.

3.4. Procedures for cleaning and drying bottles before coating

a. Don PPE, including reusable nitrile cleaning gloves.

b. Soak aspirator tubes post-insecticide bioassay in warm water with dish soap for 24 hours.

c. Soak all bottles and caps post-insecticide bioassay in hot water with dish soap for 24 hours

d. Drain water and rinse until no soap residue remains. Soak all aspirator tubes and bottle caps in tap water or deionized (DI) water if available, for an additional 24 hours and allow to air dry in a drying rack.

e. Inspect bottles to ensure they are dry. Bottles are dry when no water droplets are observed in the bottles and caps.

f. To assist in drying, bottles can be placed in an oven (50°C) for 15–20 min. If there is no oven, leave bottles to dry completely at room temperature or in the sun, with the caps off. In humid conditions, bottles can be left to dry with caps off overnight or longer.

g. Cap bottles. Bottles can be stored in their original boxes.

Bottle Wash Evaluation

a. Using a mouth aspirator, transfer 5-10 insecticide susceptible adult female mosquitoes into the bottle being evaluated.

b. Cap the bottle.

c. Set a timer for two hours.

d. If no knockdown or mortality is observed after two hours, the wash method was sufficient. If knockdown or mortality occurs, then the wash method was not sufficient and should be repeated or intensified.

3.5. Marking of bottles

a. Since the bottles can be reused, consider using a piece of masking tape on the bottles and caps for marking them instead of writing directly on the bottles and caps (Figure 2). Marking both the cap and the bottle is vitally important because the inside of the entire bottle will be coated, including the inside of the cap.

b. Mark one bottle and its cap as control.

c. Mark the other four bottles and caps with the replicate number (1-4). If running multiple insecticides, also include the type of insecticide and the bottle treatment date. If more than one concentration of an insecticide is being tested, also include the corresponding insecticide concentration.

Figure 2: Labeling bottles and caps. *Accessible explanation on page 28.*

3.6. Bottle coating

a. Make sure that bottles and caps are completely dry.

b. Remove caps from the bottles.

c. If using disposable pipettes, label one pipette as 'solvent only' for the control bottle, and another pipette as 'insecticide solution' for the test bottles.

d. Add 1 ml of acetone or ethanol to the control bottle and put the cap back on tightly.

e. In the first test bottle, add the correct volume of the prepared insecticide stock solution to achieve the desired diagnostic dose (Table 1). For example, if the stock solution has the same concentration of insecticide per ml as the diagnostic dose, add 1 ml of stock solution to the bottle. Put the cap back on tightly.

f. Repeat **Step e** with the other three test bottles.

g. Swirl the contents inside the bottle so that the bottom is coated (Figure 3).

h. Invert the bottle and swirl to coat the inside of the cap (Figure 4).

i. Place the bottle on its side to let the contents pool. Gently rotate while rocking the bottle gently so that the sides all the way around are coated.

j. Repeat this for all the test bottles and the control bottle (Figure 5).

k. Remove the caps and continue rolling bottles on their side until all visible signs of the liquid are gone from inside and the bottles are completely dry. A bottle roller can also be used to roll multiple bottles simultaneously (Figure 6).

l. Leave bottles on their sides with caps off and cover with something that will keep them protected from light.

m. If bottles are not used right away, store bottles in a dark place (such as a drawer) with the caps off to avoid moisture build-up. If shipping pre-coated bottles, ship the bottles with the caps on. More information on the storage of coated bottles is given in Section 4.3.

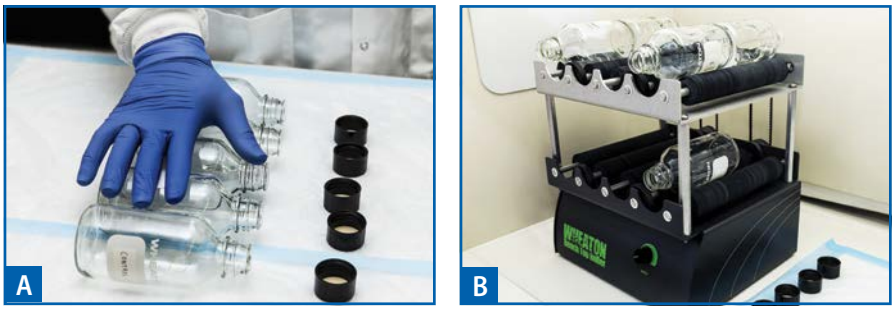
Accessible explanation for figures 3–5 on page 28, for figures 6A and 6B on page 29.

Figure 3: Coating the bottom of the bottle.

Figure 4: Coating the inside of the cap.

Figure 5: Coating the sides of the bottle.

Figure 6: Drying the bottles manually (A) or with a bottle roller (B).



4. CDC bottle bioassay method

4.1. General considerations

a. Use a filter in the aspirator to avoid inhaling mosquitoes or insect fragments.

b. Blow gently to expel the mosquitoes into the bottles. If you blow too hard, the mosquitoes can be damaged by hitting the sides of the bottle and may be killed as a result of physical damage rather than the insecticide.

c. Be careful not to touch the inside of the bottle with the aspirator, as this may contaminate the aspirator.

d. Ambient temperature and relative humidity can affect bioassay results. All efforts should be made to ensure the bioassays are conducted under controlled conditions, ideally at 27°C +/−2°C and 75% +/−10% relative humidity.

4.2. Bioassay procedure

The bioassay can be performed with the bottles in an upright position or with the bottles lying on their sides. The important thing is to be consistent and follow the same procedure each time.

a. Using an aspirator, introduce 10–25 female mosquitoes into the control bottle and quickly tighten the lid.

b. Introduce 25 female mosquitoes into each test bottle and quickly tighten the lids (Figure 7). The exact number does not have to be precise, although at least 100 mosquitoes should be tested from any given population in order to say with confidence that the results of the bioassay reflect the status of the overall population.

c. Start a timer.

d. Be sure to examine the bottles at Time 0 and count the number of dead and/or live mosquitoes. If you find dead mosquitoes at Time 0, make a note of them on the form (Appendix 3).

e. Record how many mosquitoes are dead or alive, whichever is easier to count, every 15 minutes until the diagnostic time (which is typically 30 minutes). To obtain additional data for time/mortality curves, you can continue to observe and count at 15-minute intervals until all are dead, or up to 2 hours (Figure 8).

f. Record all data on the reporting form (Appendix 3).

g. Graph the total percent mortality (Y-axis) against time (X-axis) for all replicates considered together using a linear scale.

h. Remember that mortality at the diagnostic time is the most critical value because it represents the threshold between susceptibility and resistance. Refer to Table 1 for diagnostic doses and times for commonly used insecticides.

i. Take into consideration mortality in the control bottle when reporting the results of the bioassay (Section 4.5). Use Abbott's formula to correct results if the mortality in the control bottle is between 3% and 20%. You should discard the bioassay results if mortality in the control bottle is 20%.

For the purpose of this bioassay, mosquitoes are considered dead if they can no longer stand. See Box 3 for more information.

Individual timers can be started when mosquitoes are introduced into each individual bottle to account for the lapse of time required to gently collect and aspirate mosquitoes into each bottle. To speed the transfer process, mosquitoes can be pre-sorted into small holding cups containing 25 mosquitoes each that can then be transferred into their corresponding bottles at the start of the bioassay. Mosquitoes alive at the diagnostic time (Table 1) represent mosquitoes resistant to the insecticide being tested. These mosquitoes may be transferred to a different container for further analysis (e.g., molecular assays). Mosquitoes alive at the end of the bioassay in the control bottle may need to be killed to get an accurate count. Mosquitoes can be killed by freezing or stunning them.

Box 3: Notes about mortality criteria.

- Mosquitoes can be categorized as "Dead" when they:
  - Cannot stand on their legs
  - Cannot fly in a coordinated manner
  - Are immobile and slide along the curvature of the bottle
  - Move legs and wings but cannot take off
  - Stand and take off briefly, but fall down immediately
- It helps to gently rotate the bottle while taking the count.
- It is easier to count the number of dead mosquitoes in the first readings of the bioassay, and it is easier to count the number of live mosquitoes when few remain alive.

Accessible explanation for following figures 7–8A and 8B on page 29.

Figure 7: Transferring mosquitoes into insecticide-coated bottles.

Figure 8: CDC bottle bioassay in progress. Bottles can be kept horizontally (A) or vertically (B).

4.3. Handling of coated bottles

More than one batch of mosquitoes can be tested in a single bottle in one day. However, the main limiting factor for reusing previously coated bottles is moisture build-up with successive introductions of mosquitoes, especially in humid environments. If the bottles are to be reused on the same day, it is necessary to leave some time (2-4 hours; maybe longer if in a humid climate) between the bioassays for the bottles to dry out (with caps off) before introducing more mosquitoes. If the bottles are to be reused the following day, bottles can be left to dry with caps off overnight at room temperature and should also be protected from direct light. **Bottles should not be dried in the oven after they have been coated with insecticide, as this can degrade the insecticide.**

If the bottles are not to be used soon after coating them with insecticide, once they are dry, they should be stored in a dark place (such as a drawer) at room temperature, with their caps off. Depending on the insecticide used, bottles can be stored for up to 5 days in this manner. The length of time bottles can be stored depends on the insecticide. DDT and organophosphate-coated bottles should be used immediately and not stored. Bottles can be coated in a central laboratory and sent for use in the field. During transport, bottles should have their caps on. Alternatively, appropriately dosed stock solutions can also be sent to the field for treating bottles.

4.4. Identification of mechanisms of resistance

If resistance is detected in a population, the mosquitoes from the bioassay can be used for further testing to detect molecular markers of resistance. Surviving mosquitoes may be easily released from bottles into a holding carton to separate them from those killed during the CDC bottle bioassay. Knowing the phenotype of the mosquitoes that undergo molecular analyses is critical—be sure to keep the survivors of the bioassay ('resistant' mosquitoes) separate from the mosquitoes that were killed ('susceptible' mosquitoes). Also ensure that all tubes are appropriately labeled so that the resistant and the susceptible individuals are readily identified. Mosquitoes to be used for molecular studies can be frozen, dried, or stored in 70% (or higher) ethanol. It may be necessary to use products like RNALater® (Applied Biosystems [Ambion], Foster City, California) to preserve samples for measurement of RNA markers associated with differential gene expression.

If additional mosquitoes remain (i.e., not all mosquitoes were used in the bioassays), biochemical assays may be conducted to quantify levels of activity of certain large enzyme families that can contribute to insecticide resistance. **It's important to note that biochemical assays should never be conducted on mosquitoes that have been exposed to an insecticide in a bioassay.** They should only be conducted immediately after killing mosquitoes by freezing the mosquitoes, or on mosquitoes that had been stored at -80°C, because the proteins can degrade rapidly.

4.5. Validity of the data

The mortality of mosquitoes in the control bottle should be zero. In most cases, mortality of up to 3% in the control bottle may be ignored. In cases where mortality is up to 20% in the control bottle, Abbott's formula can be used to correct the findings (see Box 4). If mortality in the control bottle is greater than 20% at the end of the bioassay, the results should be discarded, and the bioassay should be repeated. However, if a particular mosquito collection is essentially irreplaceable and the bioassay cannot be repeated, Abbott's formula can be considered even when control mortality is >20%.

Accessible explanation for the following 'Box 4: Abbott's formula' on page 30.

Box 4: Abbott's formula.

$$\text{Corrected mortality} = \frac{(\text{mortality in test bottles [\%]} - \text{mortality in control bottle [\%]}) \times 100}{(100\% - \text{mortality in control bottle [\%]})}$$

For example: If mortality in test bottles is 50% at diagnostic time and control mortality is 10%, the corrected mortality is $[(50\%-10\%) / (100\%-10\%)] \times 100 = 44.4\%$

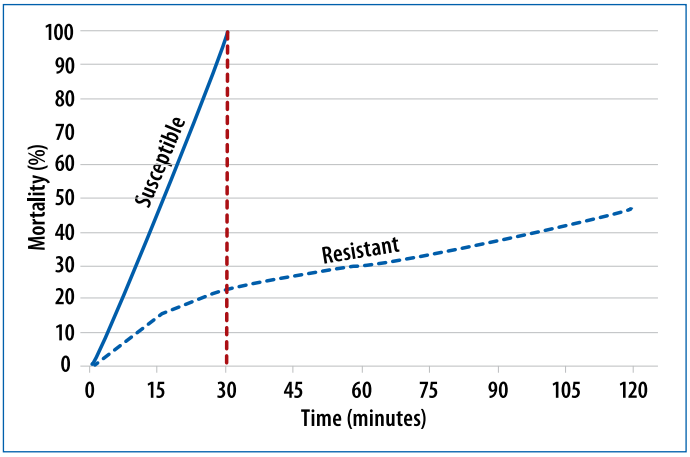
Note: In cases of 100% mortality in test bottles, Abbott's formula has no effect.
For example: $[(100\%-10\%)/(100\%-10\%)] \times 100 = 100\%$ corrected mortality

4.6. Interpretation of results

If mosquitoes survive in the insecticide-coated bottles beyond the diagnostic time, these survivors represent a proportion of the population that has something preventing the insecticide from efficiently reaching its target site and acting. In other words, they have some degree of resistance. In the example shown in Figure 9, all mosquitoes that died before the diagnostic time when exposed to insecticide-coated bottles were susceptible. Tested mosquitoes surviving beyond the diagnostic time are assumed to have some degree of resistance. In the example, only 23% of the test population was susceptible. Recommendations for interpretation of bioassay data are shown in Box 5. The most important information is the mortality at the diagnostic time, but the bioassay can be carried out beyond the diagnostic time to obtain a more complete mortality curve. When resistance is confirmed, pre-emptive action should be taken to manage insecticide resistance and to ensure that the effectiveness of insecticides used for vector control is preserved.

Figure 9: Determination of resistance at a diagnostic time of 30 minutes.

Accessible explanation on page 29.



Box 5: Interpretation of bioassay data.

- 98%–100% mortality at the diagnostic time indicates susceptibility.
- 90%–97% mortality is suggestive of resistance and further investigation is recommended.
- If mortality is less than 90%, the population is considered resistant. Further investigation of the mechanisms and intensity of resistance is recommended.

5. Intensity bioassay

5.1 Background

Historically, the major methods of mosquito bioassays (principally the WHO tube assay and the CDC bottle assay) have focused on determining the frequency of insecticide resistance in a mosquito population following exposure to a discriminating (also referred to as diagnostic) dose of insecticide at a pre-determined diagnostic period of time. However, resistance frequency data alone provides limited evidence to inform the crucial decisions that must be made in procurement and deployment strategies for public health insecticides. Determining the intensity of resistance offers practical information of significance to decision-making. For example, let us suppose that two populations of anophelines (in different areas within a country) show a resistance frequency of 25% in any of the accepted bioassay formats. If none of the resistant mosquitoes in site A can survive exposure to twice the diagnostic dose of a particular insecticide but those at site B have 15% that survive 5 or even 10 times the diagnostic dose at the diagnostic time, the options for vector control at both sites may look quite different.

5.2 Resistance Intensity Bioassay

The simplest resistance intensity bioassay uses bottles (one per dose) treated with 1, 2, 5 and 10 times the diagnostic dose of insecticide plus a control. The diagnostic time is not altered. More mosquitoes would be needed for a confident assessment of resistance intensity at a particular site but note the qualitative value of knowing if even one or two mosquitoes can survive at the 5x and 10x doses. That would serve as an alert that a particular site merits closer surveillance.

Given a large number of available mosquitoes, up to 25 mosquitoes per bottle can give more reliable information on resistance intensity, and ideally, four replicates of each dosage can be run. If resistance has been detected at the diagnostic dose using the conventional bottle bioassay, the next step would be to screen the population for resistance intensity using 2x the diagnostic dose. If mosquitoes survive 2x the diagnostic dose, then the assay should be run at 5x the diagnostic dose. If mosquitoes survive 5x the diagnostic dose, the population should be screened using 10x the diagnostic dose. Note that higher dosages than 10x may be needed to establish maximum intensity levels in some mosquito populations, but limitations may exist regarding the ability to treat bottles with higher doses of some insecticides due to crystallization.

6. CDC bottle bioassay with synergists

6.1. Background

The CDC bottle bioassay using bottles coated with a single insecticide provides information on the frequency of insecticide resistance in a given population to a particular insecticide. Once resistance is detected, or at least suspected, it's important to determine which alternative insecticides are likely to still be effective and not compromised by cross resistance. This requires knowledge of the resistance mechanism(s) in the population, which is often determined through biochemical (microplate) assays or molecular methods. A rapid and inexpensive alternative to assess metabolic resistance mechanisms is to use the CDC bottle bioassay with synergists. Synergists are inhibitors of insecticide detoxification enzymes. Synergists are available for several large enzyme families important to the metabolic detoxification of insecticides: esterases, oxidases, and glutathione S-transferases.

Synergists act by restoring susceptibility if detoxification enzymes play a key role in resistance to a particular insecticide (Figures 10a and 10b). Hypothetical data for resistant and susceptible populations are shown (Figure 10a). Once a synergist is used on the resistant population, one of three things might happen (Figure 10b):

a. Susceptibility to the insecticide is fully restored (time-mortality line A), which suggests that the mechanism inhibited by that synergist is playing a major role in conferring resistance to that particular insecticide

b. Susceptibility to the insecticide is partially restored (time-mortality line B). This suggests that the mechanism inhibited by that synergist is contributing to the resistance, but it is not the only mechanism involved.

c. Susceptibility to the insecticide is unaffected (time-mortality line C). This indicates that the mechanism inhibited by the synergist is not contributing to the resistance.

The proportion of susceptibility restored in a population through the use of synergists provides an estimate of the proportion of resistance that is attributable to other mechanisms, such as target site insensitivity. Further biochemical and/or molecular assays can confirm the prevalence of target site mechanisms, such as kdr (mutations on the voltage-gated sodium channel gene) or insensitive acetylcholinesterase.

Figures 10a and 10b. Effects of synergists on resistant vector populations.

Figure 10a shows data for a population of resistant vectors compared to a susceptible population. Figure 10b shows the three possible outcomes of synergist pre-exposure: Line A, susceptibility to the insecticide is fully restored; Line B, susceptibility to the insecticide is partially restored; and Line C, susceptibility to the insecticide is unaffected.

Accessible explanation of figures 10a and 10b on page 30.

Figure 10a.

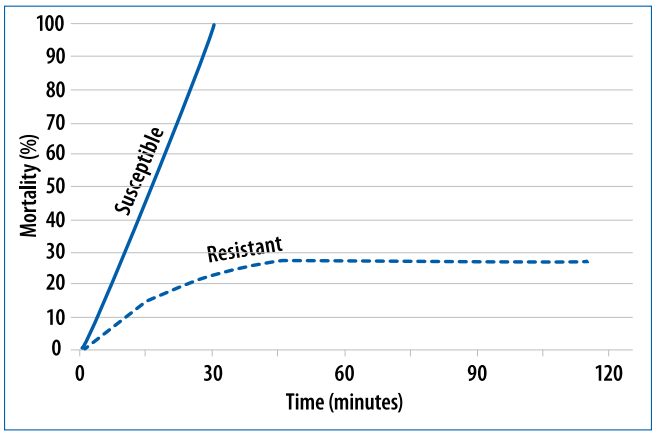
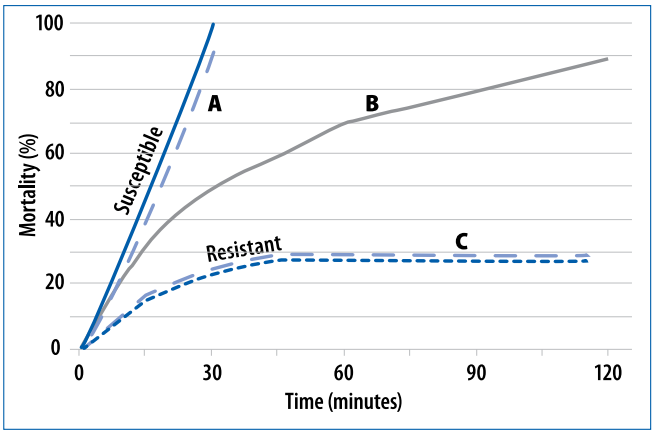


Figure 10b.



6.2. Use of synergists

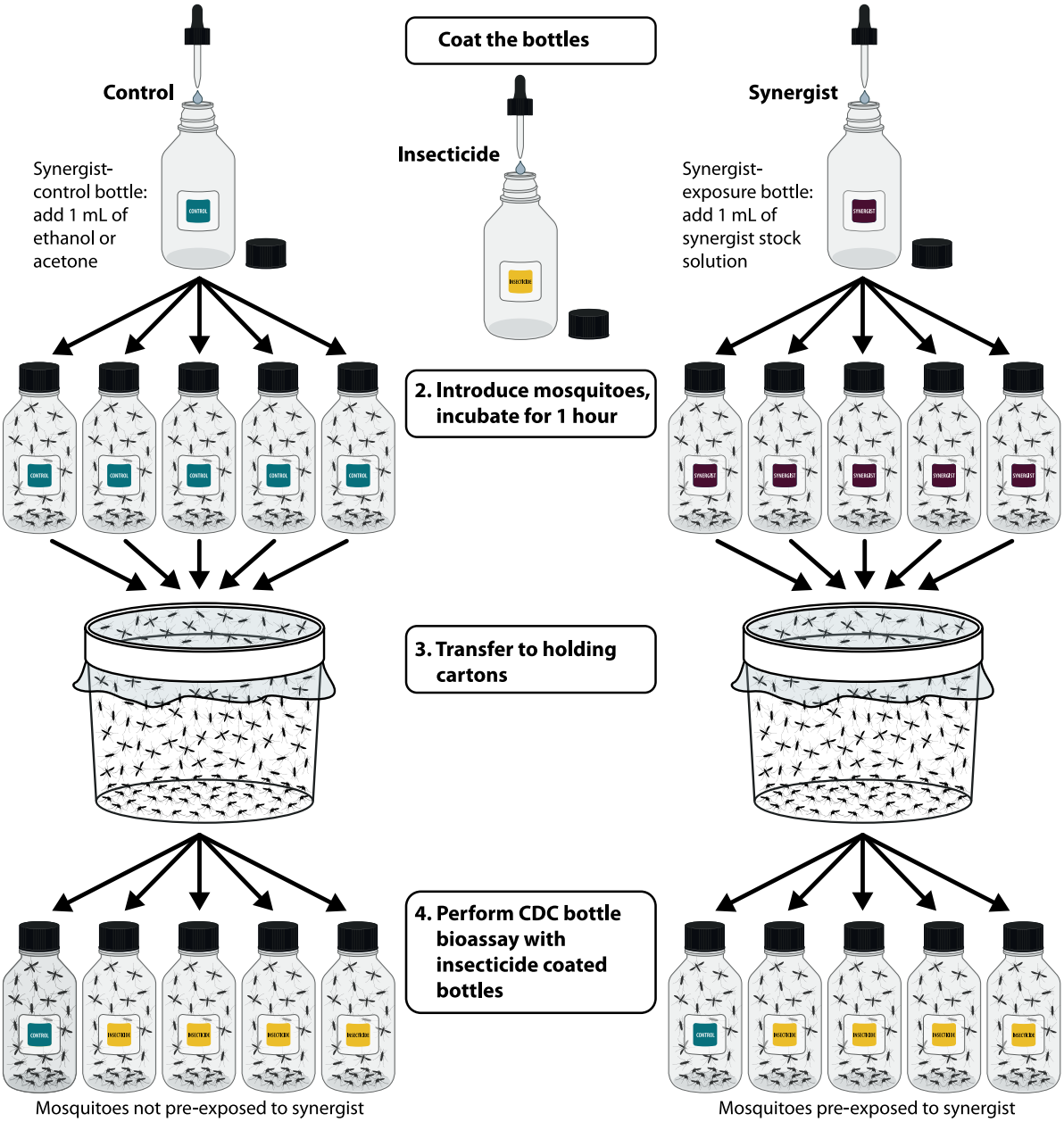
Commonly used synergists in conjunction with the CDC bottle bioassay include:

- Piperonyl butoxide (PBO), which inhibits oxidase activity
- S.S.S-tributlyphosphorotrithioate (DEF), which inhibits esterase activity
- Ethacrynic acid (EA), diethyl maleate (DM), and chlorfenethol (CF), which inhibit glutathione S-transferase activity

Testing mosquitoes with synergists is a two-step procedure. Mosquitoes are first exposed to the synergist for 1 hour and then exposed to the insecticide of interest using the CDC bottle bioassay. A schematic representation of performing a bioassay with a synergist is shown in Figure 11.

Figure 11. Performing the CDC bottle bioassay with synergists.

Accessible explanation of figure on page 30.



6.3. Preparation of bottles for synergist bioassays

a. Prepare the synergist stock solution by diluting the appropriate amount of synergist in acetone or technical grade ethanol to be able to coat the bottles with the concentrations shown in Table 3. To make these stock solutions, follow the same procedure described for making insecticide stock solutions (Section 3.2). For example, to obtain a concentration of 100 µg/bottle of piperonyl butoxide (PBO), dissolve 100 mg in enough acetone or absolute ethanol to make 1 liter of solution. Each 1 ml of this solution will contain 100 µg of PBO.

b. Mark one bottle and its cap as the synergist-control bottle (without synergist).

c. Mark a second bottle and its cap to be the synergist-exposure bottle.

d. Add 1 ml of acetone or ethanol to the synergist-control bottle and put the cap back on tightly.

e. Add 1 ml of the synergist stock solution to the synergist-exposure bottle and put the cap on back tightly.

f. Coat the bottles, remove the caps, and let the bottles dry as described in Section 3.6.

g. Prepare two sets of insecticide-treated bottles to run the CDC bottle bioassay (Section 3.6).

Table 3: Synergist concentrations used in the CDC bottle bioassay.

Synergist	Synergist concentration (µg/bottle)
Chlorfenethol (CF)	80
Diethyl maleate (DM)	80
Ethacrynic acid (EA)	80
Piperonyl butoxide (PBO)	100
S.S.S-tributlyphosphorotrithioate (DEF)	125

6.4. Procedure for the CDC bottle bioassay with a synergist

a. Introduce equal numbers of mosquitoes into the synergist-control bottles and into the synergist-exposure bottles (about 25 mosquitoes in each bottle).

b. Keep the mosquitoes in the bottles for 1 hour to allow the synergist to act.

c. After the 1-hour exposure is complete, transfer the mosquitoes to their respective holding cartons, one for the non-exposed control mosquitoes and another for the synergist-exposed mosquitoes. This makes it easier to subsequently transfer the mosquitoes into the insecticide-treated bottles.

d. Perform the CDC bottle bioassay as described in Section 4.2 using one set of insecticide-coated bottles (one control and four test bottles) for the synergist-control mosquitoes and another set (one control and four test bottles) for the synergist-exposed mosquitoes.

6.5. Interpretation of data from synergist bioassays

Section 6.1, 6.2, and Figures 10a and 10b provide information on how to interpret the results of the CDC bottle bioassay using synergists. Resistance that cannot be attributed to one of the detoxification mechanisms after all synergists have been tested is likely to be due to target site mechanisms, such as kdr or insensitive acetylcholinesterase, or other physiological factors.

7. Resistance surveillance

7.1. Background

Although resistance data are often collected as part of vector control programs, this is often not done as routinely or systematically as would be optimal. The CDC bottle bioassay is an instrument to collect information to support an insecticide resistance surveillance system. Resistance data are most valuable when collected routinely, in the same sites, over time to allow for comparisons and for monitoring of trends.

It is important to consider how information collected as part of an insecticide resistance surveillance system will be used. Most malaria control programs assess the efficacy of their vector control actions by monitoring the impact on malaria incidence, as well as through the measurement of entomological indicators. Insecticide resistance data can be integrated into routine entomological surveillance in order to guide vector control and insecticide resistance mitigation strategies.

7.2. Guiding principles

Multiple genetic, biologic, environmental, and operational factors can influence the development of insecticide resistance. Resistance can be highly focal, with patterns differing over only a few kilometers for *Anopheles* or even by city blocks for *Aedes*. As a guiding principle, vector control programs should aim to monitor susceptibility in areas that receive (or are likely to receive) regular insecticide-based interventions and at a geographical scale that would allow for the tailoring of vector control actions based on susceptibility status. In general terms, resistance surveillance should be conducted in areas where disease transmission is a concern and where insecticide-based control measures are used or planned, ideally before purchase of insecticide-based products. Even if only one or a few sites can be monitored, this is preferable to having no surveillance sites. In addition, efforts should be made to operate sites for at least a few years, since comparative data provide the most meaningful information.

Ideally, each site should be monitored at least once a year. Where control efforts are seasonal, it may be useful to monitor at the beginning and at the end of the control season. If several vectors in the area are seasonal, the resistance testing schedule should be adjusted to the species of interest.

It is also important to consider that it may be necessary to identify resistance mechanisms once resistance is detected, whether using the CDC bottle bioassay with synergists, or biochemical and/or molecular methods. Cross-resistance to multiple insecticides can arise when mechanisms are shared, so insecticide choice is further informed when information is available regarding the specific mechanism(s) of resistance.

Finally, some countries have found it useful to centralize activities related to insecticide resistance surveillance. A central reference laboratory can provide technical support, quality assurance, and serve as the central repository of resistance data. When bioassays are conducted under a decentralized model, a central reference laboratory can play an important role in training, provision of supplies, and additional analyses, such as the use of biochemical and molecular methods for determination of resistance mechanisms.

8. Bibliography

1. Brogdon, WG and McAllister JC, 1998. Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. J. Am. Mosq. Control Assoc. 14(2):159–64.
2. National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC). Evaluating mosquitoes for insecticide resistance: Web-based instruction. Available from: http://www.cdc.gov/malaria.
3. World Health Organization, 2022. Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions. Available from: https://www.who.int/publications/i/item/9789240051089

Appendix 1. Frequently asked questions (FAQs)

1. **What happens if there are not enough mosquitoes for a complete bioassay?**
   When the number of mosquitoes available from a population is insufficient for a full bioassay (typically 100 mosquitoes exposed to the insecticide-treated bottles and at least 10 in the control bottle), you can reduce the number of bottles to be tested, but each bioassay must always be run with a control. In order to draw conclusions regarding the resistance status of a population, at least 100 mosquitoes from that population should be phenotyped. Mosquitoes can be tested on multiple days to achieve the right numbers. If this is not possible to achieve, the results should be interpreted with caution.

2. **Should some bottles be designated solely as control bottles?**
   No, some bottles should not be designated as control bottles. Bottles should randomly be assigned as test or control bottles. This will provide an additional quality control to the adequacy of the washing procedure.

3. **Can male mosquitoes be used for the control bottle?**
   No, males should not be used for the control bottles. Some resistance mechanisms are sex- linked, and one can be misled by using males in the control. In addition, female mosquitoes are the primary targets of vector control.

4. **How can mosquitoes be introduced into the bottle without letting some escape?**
   Some people have found it useful to employ a piece of fabric or cotton wool held against the aspirator tube at the top of the bottle to close any gaps as the mosquitoes are being introduced into the bottles. As the aspirator is withdrawn after the mosquitoes are introduced, the cotton wool can be used to cover the bottle top until the bottle cap is put in place. In our experience, a gentle yet swift and decisive puff of air will introduce mosquitoes without escape. Attempting to introduce mosquitoes into a bottle more than once increases the likelihood that some may escape.

5. **If testing mosquitoes directly collected from the field, what happens if there are fed and unfed mosquitoes among the field-collected mosquitoes to be used in the bioassay?**
   A collection of mosquitoes from the field may contain female mosquitoes in various physiological states, e.g., fed and unfed mosquitoes. There are two ways that this can be dealt with. First, mosquitoes can be tested together regardless of their physiological state, although the mixed background of the population should be noted when interpreting results. Alternatively, mosquitoes can be held for one or two days for the blood meal to be digested and then used for the bioassay.

Appendix 2. Diagnostic doses and CDC bottle bioassay calibration

It is assumed that resistance is present if exposure to a diagnostic dose is survived by members of a test population at a predetermined diagnostic time. For some mosquito genera, diagnostic doses and diagnostic times for several insecticides have already been determined. However, if this information is not available, the diagnostic dose and the diagnostic time will need to be defined for a given insecticide and for each main vector species that is to be monitored. To determine the diagnostic dose and the diagnostic time for use in the CDC bottle bioassay, the assay will have to be calibrated.

Calibration assay

Population: The first step is to select a susceptible mosquito population to use in the calibration. If such a population is not available, it is possible to use a local mosquito population from the area where the chemical vector control measures are to be applied. This population will serve as the reference point against which all future populations can be compared.

Diagnostic time: For practical reasons, the diagnostic time should be between 30 and 60 minutes.

Diagnostic dose: The diagnostic dose will be a dose of insecticide that can kill 100% of susceptible mosquitoes within 30 to 60 minutes and that is below the saturation point. To determine possible diagnostic doses, first prepare bottles with a wide range of different concentrations of insecticide per bottle. Using each of these different bottles, run separate CDC bottle bioassays on 25 mosquitoes of the susceptible population to determine the upper limit of the diagnostic dose, which is the saturation point. The saturation point is defined as the concentration above which the time to kill 100% of the mosquitoes remains the same even if the concentration increases. See a more detailed explanation on how to determine the saturation point below.

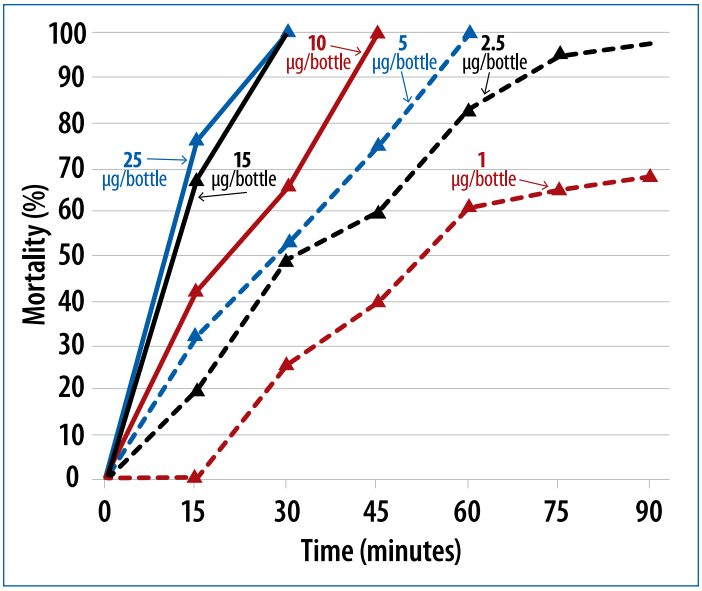
Interpretation of calibration data

Graphing the results of the calibration assay will show that the time-mortality line becomes straighter, steeper, and closer to the Y-axis as the insecticide concentration increases (Figure A1). This means that by increasing the insecticide concentration, the time-mortality line will reach a point where increasing the concentration of insecticide will not kill all mosquitoes any faster. In the example below, 15 µg/bottle is the toxicological saturation point for the insecticide entering the mosquito and reaching its target. Increasing the concentration to 25 µg/bottle does not cause the insecticide to penetrate the mosquito, reach the target site, and kill the mosquito any faster. Therefore, 15 µg/bottle is the saturation point and the maximum concentration to use as the diagnostic dose. Otherwise, there is a risk that resistant mosquitoes will be killed by doses higher than the saturation point and then be recorded as susceptible, i.e., false negatives for resistance.

A slightly smaller concentration compared to the toxic saturation point will kill mosquitoes in an amount of time perhaps more convenient for the user (e.g., 30 to 60 minutes). So, it is possible to choose a lower diagnostic dose that kills 100% of mosquitoes within 30 to 60 minutes. Although several different pairs of diagnostic doses and diagnostic times can give interpretable results, it is necessary to consistently use the same diagnostic dose and diagnostic time for that particular insecticide on that particular vector in future assays to allow for comparability over time.

Figure A1: Determining diagnostic doses and diagnostic times.

Accessible explanation on page 30.



In the example shown, 15 µg/bottle is the saturation point because a higher dose did not decrease the time for 100% of susceptible mosquitoes to be killed. Concentrations <5 µg/bottle take 60 minutes or more to kill 100% of susceptible mosquitoes. The doses between 5 and 15 µg/bottle are in the usable range for detecting resistance, and the diagnostic time for each of these concentrations is the time at which 100% of mosquitoes were killed. So, for example, a diagnostic dose of 10 µg/bottle and diagnostic time of 45 min could be used.

Appendix 3. Example CDC bottle bioassay data recording form

Date (mm/dd/yy): ..... / ..... / ..... Mosquito species: .....

Insecticide: .....

Date of Bottle Treatment (mm/dd/yy): ..... / ..... / ..... Number of times used: .....

Diagnostic dose: ..... Diagnostic time: .....

Location of mosquito collection: .....

Generation: .....

Temperature: ..... Relative humidity: .....

Operator Name: .....

Table for recording alive or dead mosquitos in individual test bottles

Time (min)	Bottle 1: Alive	Bottle 1: Dead	Bottle 2: Alive	Bottle 2: Dead	Bottle 3: Alive	Bottle 3: Dead	Bottle 4: Alive	Bottle 4: Dead
0								
15								
30								
45								
Total in bottle								

Table for recording combined test bottles results

Time (min)	Total dead	Total tested	% Dead
0			
15			
30			
45			
Total in bottle			

Table for recording control bottle results

Time (min)	Total dead	Total tested	% Dead
0			
15			
30			
45			
Total in bottle			

Comments:

Appendix 4. Explanation of Figures for Accessibility

Figure 1: Example of materials and reagents for the CDC bottle bioassay (Page 6)

Overview— Shows an example of the materials that are necessary to run the CDC Bottle Bioassay.

Description—Photograph of items that are included on the Bottle Bioassay Kit. Shows from top left to right: 5 250-ml Wheaton bottles with screw lids, 1 timer, 2 aliquots of insecticides, 1 Aspirator apparatus, 25 50-ml falcon tubes, placed on top of a blue lab pad.

Presentation—Color Photograph (Return to Page 6)

Figure 2: Labeling bottles and caps. (Page 12)

Overview— Shows an example of how to label the bottles and their caps.

Description— Photograph of five 250ml Wheaton Bottles with screw lids on. Each bottle and its cap has a corresponding white tape label in the following order: Control, 1, 2, 3, 4. There is also one aspirator apparatus shown in the photograph. Everything is placed on top of a blue lab pad, to prevent contamination of the surface area.

Presentation— Color photograph. (Return to Page 12)

Figure 3: Coating the bottom of the bottle. (Page 13)

Overview— Shows the operator, wearing proper PPE, lab coat and gloves are shown.
The operator is coating the bottom of a bottle.

Description—The operator is handling a test bottle labeled as number 1, holding it by the tightly closed cap, and rotating the liquid in the bottom surface of the bottle, making sure it covers all the area.

Presentation— Color photograph. (Return to Page 13)

Figure 4: Coating the top of the bottle. (Page 13)

Overview— Shows the operator, wearing proper PPE, lab coat and gloves are shown. The operator is coating the top of a bottle.

Description— The operator is handling a test bottle labeled as number 1, holding it by the tightly closed cap, and inverting the bottle, making the liquid in the bottom to go to the top, where the cap is. The operator rotates the bottle by the cap, making sure the insecticide covers all the area.

Presentation— Color photograph. (Return to Page 13)

Figure 5: Coating the sides of the bottle (Page 13)

Overview— Shows the operator, wearing proper PPE, lab coat and gloves are shown. The operator is coating the sides of a bottle.

Description— The operator is handling a test bottle labeled as number 1, holding it by the tightly closed cap and the bottom, now the bottle is parallel to the working table. The operator is making the insecticide run sideways by titling the bottle slowly in an up and down motion, making sure it covers all the glass surface area.

Presentation— Color photograph. (Return to Page 13)

Figure 6: Drying the bottles manually (A). (Page 13)

Overview— Shows the operator, wearing proper PPE, lab coat and gloves are shown. The operator is drying the bottles.

Description— After coating the control bottle and test bottles 2, 3, and 4, the operator removed the caps, and placed the bottles on their sides, setting the caps aside to dry. The bottles are placed horizontally on the working table and the operator is rolling them to allow the bottles to dry uniformly while the insecticide still coats the glass walls. The operator can roll the 5 bottles with the whole hand back and forth. Depending on the temperature and humidity of the room, the drying process can be shorter or longer.

Presentation— Color photograph. (Return to Page 13)

Figure 6: Drying the bottles with a bottle roller (B). (Page 13)

Overview— Shows a bottle roller, inside a chemical hood drying the bottles.

Description— After coating the control bottle and test bottles 2, 3, and 4, the operator removed the caps, and placed the bottles horizontally on the bottle roller and turned the roller on. The bottle roller will allow the bottles to dry uniformly while the insecticide still coats the glass walls, without the need of an operator rotating the bottles. Depending on the temperature and humidity of the room, the drying process can be shorter or longer.

Presentation— Color photograph. (Return to Page 13)

Figure 7: Transferring mosquitoes into insecticide-coated bottles. (Page 15)

Overview— Shows the operator transferring live mosquitoes with a mouth aspirator, wearing proper PPE, lab coat and gloves are shown.

Description— The operator is handling live mosquitoes using a mouth aspirator. The appropriate number of mosquitoes (25) have been loaded into the aspirator and then carefully transferred into bottle labeled "1". The cap, also labeled "1" is ready to cover the bottle and prevent mosquitoes from flying away.

Presentation— Color photograph. (Return to Page 15)

Figure 8: CDC bottle bioassay in progress. Bottles can be kept horizontally (A) or vertically (B). (Page 15)

Overview— Shows an example of the CDC bottle assay in progress.

Description— Photograph of five 250ml Wheaton bottles with screw lids on. Each bottle and its cap has a corresponding white tape label in the following order: Control, 1, 2, 3, 4. Photograph A shows how the mosquitoes can be counted while the bottles lie on the working table, horizontally. Photograph B shows how the mosquitoes can be counted while the bottles stand vertically on the working table.

Presentation— Color photograph. (Return to Page 15)

Figure 9: Determination of resistance at a diagnostic time of 30 minutes (Page 17)

Overview— The figure shows a graph for the determination of resistance at 30 minutes.

Description— The X axis shows time in minutes and the Y axis shows the mortality percentage, tracking two populations. The susceptible population, represented by the continuous line, shows the mortality increasing rapidly through time, to the point where 100% of the individuals tested are dead by 30 minutes. The resistant population, represented by the dotted line, shows the mortality climbing slowly and reaching only 20% mortality by the 30-minute mark, which is the diagnostic time for most insecticides in the CDC Bottle Bioassay. The time for 30 minutes is marked in red.

Presentation— Graph. (Retrurn to Page 17)

Figures 10a and 10b. Effects of synergists on resistant vector populations (Page 19)

Overview— The figure shows two graphs for the effects of synergists on resistant vector populations.

Description— Figure 10a. (Similar to Figure 9) The X axis shows time in minutes and the Y axis shows the mortality percentage, tracking two possible outcomes for vector populations. The susceptible population, represented by continuous line A, shows the mortality increasing rapidly through time, to the point where 100% of the individuals tested are dead by 30 minutes. The resistant population, represented by dotted line B, shows the mortality of individuals climbing slowly and reaching only 20% mortality by the 30-minute mark, which is the diagnostic time for most insecticides in the CDC Bottle Bioassay.

Figure 10b. Tracks 3 possible outcomes for resistant vector populations pre-exposed to a synergist. Line A, shows the susceptibility to the insecticide fully restored; Line B, susceptibility to the insecticide is partially restored; and Line C, susceptibility to the insecticide is unaffected.

Presentation— Two graphs. (Return to Page 19)

Figure 11. Performing the CDC bottle bioassay with synergists (Page 20)

Overview— Diagram on how to perform CDC bottle bioassays pre-exposing the mosquitoes to a synergist.

Description— Diagram separated into two columns, left and right, because processes need to happen in parallel. Shows diagrams of empty coated bottles, holding cartons and coated bottles with mosquitoes.

Left side shows the step for the Control Bottle; Right side shows the steps for Synergist Exposure.

LEFT SIDE: 1. Coat the bottles with 1 ml of ethanol or acetone. 2. Introduce mosquitoes and incubate for 1 hour. 3. Transfer mosquitoes to holding cages or cartons. 4. Perform the bottle assay with bottles that have been coated with the insecticide being tested.

RIGHT SIDE: 1. Coat the bottles with 1 ml of synergist. 2. Introduce mosquitoes and incubate for 1 hour. 3. Transfer mosquitoes to holding cages or cartons. 4. Perform the bottle assay with bottles that have been coated with the insecticide being tested.

Presentation— Two charts. (Return to Page 20)

Figure A1: Determining diagnostic doses and diagnostic times (Page 26)

Overview— Graph of determining diagnostic doses and diagnostic times.

Description— In the example shown, 15 µg/bottle is the saturation point because a higher dose did not decrease the time for 100% of susceptible mosquitoes to be killed. Concentrations <5 µg/bottle take 60 minutes or more to kill 100% of susceptible mosquitoes. The doses between 5 and 15 µg/bottle (shaded area) are in the usable range for detecting resistance, and the diagnostic time for each of these concentrations is the time at which 100% of mosquitoes were killed. For example, a diagnostic dose of 10 µg/bottle and diagnostic time of 45 min could be used.

Presentation— Graph. (Return to Page 26)

Box 4: Abbott's formula.

Corrected mortality = (mortality in test bottles [%] minus mortality in control bottle [%]) divided by (100% minus mortality in control bottle [%]) result multiplied by 100.

For example: If mortality in test bottles is 50% at diagnostic time and control mortality is 10%, the corrected mortality is [(50%–10%) divided by (100%–10%)] result multiplied by 100 = 44.4%.

Note: In cases of 100% mortality in test bottles, Abbott's formula has no effect.

For example: [(100%–10%) divided by (100%–10%)] result multiplied by 100 = 100% corrected mortality.

Presentation— Formulas. (Return to Page 16)

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