

## Standard Operating Procedure (SOP)

### Bacterial Larvicide Bioassay

(i.e. *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*)

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#### Background

The generalized, internationally accepted method for determination of bacterial larvicide (WHO monograph = BL) active ingredient content is the bioassay of activity towards mosquito larvae. The potency of a given BL product is determined per biopotency, comparing mosquito larval mortality produced by the product under test with the mortality produced by a corresponding reference standard (results are expressed as international toxic units (ITU)/mg product for *Bacillus thuringiensis* subsp. *israelensis* (Bti) based products and results are expressed as Bs ITU/mg for *Bacillus sphaericus* (Bs) based products per WHOPES guidelines).

#### A. *Bacillus thuringiensis* subsp. *israelensis*

1. Bioassays are conducted with actively feeding L4 larvae of *Aedes aegypti*. Results expressed as international toxic units (ITU)/mg product, relative to reference Bti strain AM65-52 material.
2. NOTE: The original reference powder recommended by WHO for this purpose, IPS82 strain 1884 from Pasteur Institute, is no longer available. Until a replacement international reference powder of Bti becomes available, a reference standard of strain AM65-52 may be obtained from Valent Biosciences LLC for the purposes of testing product compliance with the specification.
  - a) The original reference standard of Bti strain AM65-52 was calibrated against IPS82 strain 1884 and was listed by the WHO upon completion of the first Bacterial Larvicide to pass the WHO Pesticide Evaluation Scheme in 2007 (reference standard Bti strain AM65-52, Lot # 82-691-W5 which had a biopotency of 7992 ITU/mg; Reference: Oct 2012 WHO specifications 770WG and 770GR for *Bacillus thuringiensis* subsp. *israelensis*, strain AM65-52). Since 2007, this lot was removed per its corresponding ‘check sample’ evaluations (i.e. lot was showing degradation); as such, the ‘check sample’ was established as the new reference standard (Bti strain AM65-52, Lot # 093-177-W502 which has a biopotency of 6388 ITU/mg).

#### B. *Bacillus sphaericus*

1. Bioassays are conducted with early L3 third instar larvae of *Culex quinquefasciatus*. Results expressed as Bs international toxic units (Bs ITU)/mg product, relative to reference strain ABTS-1743 material. NOTE: The only reference standard currently available and listed by the WHO is Valent BioSciences LLC Bs strain ABTS-1743, Lot # 089-273-W501, which has a biopotency of 1639 Bs ITU/mg (Ref: April 2016 WHO specification 770 + 978GR for *Bacillus thuringiensis* subsp. *israelensis*, strain AM65-52 + *Bacillus sphaericus*, strain ABTS-1743).
2. Prior to the first global introduction of a commercial *Bacillus sphaericus* based product in the 1990s, it was necessary for a new biopotency method to be established for quality control. Unfortunately, the internationally accepted Bti bioassay method developed in the 1980s utilized *Aedes aegypti* larvae, a species that is not susceptible to Bs (sometimes referred to as “refractory” towards Bs). As such, a new bioassay method using *Culex quinquefasciatus* was developed (unit identifier = BsITU/mg) and has been used by the international community for Bs based larvicides for over 25 years. *Culex quinquefasciatus* is recognized as the standard species for assessing potency of Bs based products internationally. As such, it requires different bioassay methods relative to Bti bioassays to account for the genus differences between *Aedes* and *Culex*. In addition, the introduction in 2008 of commercial Bti + Bs based products globally (now registered in Brazil, Nigeria, Turkey, European Union and the United States; others pending), international regulatory authorities have agreed that only a single bioassay test should be utilized (reference Bti + Bs labels)

and that the most relevant test for this combination product is the *Culex quinquefasciatus* bioassay (unit identifier = BsITU/mg). Note that select Bti + Bs products are “true combinations” at the micro/active ingredient level and are not simply a mixture of separate commercial products that are “tank mixed”. In other words, the active ingredients are “fused” at the micron level and cannot be separated in a bioassay analysis. Since *Culex quinquefasciatus* is susceptible to both Bti and Bs, then it stands to reason (position is supported by the international regulatory community) to establish this species/protocol as the reference assay for Bti + Bs combinations.

## 1. Purpose

The purpose of this document is to describe the bioassay procedure along with data generation and analysis for determining the biopotency of 1) *Bacillus thuringiensis* subsp. *israelensis* based-products using *Aedes aegypti* mosquitoes and 2) *Bacillus sphaericus* based-products using *Culex quinquefasciatus* mosquitoes.

## 2. Scope

This SOP supports testing of Bacterial Larvicides used to control mosquito larvae.

## 3. Definitions

- a. **Acceptance criteria** = The “gold” standards of the bioassays used to determine whether a test sample is sufficiently similar in relative potency to the reference standard to be approved as quality product. It states that the estimated LC50’s should be between the second lowest and second highest pesticide concentration levels. Also, the mean estimated potency should have a coefficient of variation (CV) of equal to or less than 15%.
- b. ***Aedes* (Ae)** refers to *Aedes aegypti*
- c. ***Culex* (Cx)** refers to *Culex quinquefasciatus*
- d. **Bioassay** = the use of living organism to measure the amount of substance (or potency), such as toxin, in an unknown sample.
- e. **BsITU** = *Bacillus sphaericus* international toxic units, standard measure of the biological activity of *Bacillus sphaericus* products.
- f. **Bti (*Bacillus thuringiensis* subsp. *israelensis*)** in which affects *Aedes aegypti* and **Bs (*Bacillus sphaericus*)** in which affects *Culex quinquefasciatus*, are two strains of bacteria that produce crystals with insecticidal properties.
- g. **Coefficient of variation (CV)** = For bioassays, a measure of % variation in the mean potency of test sample replicates. This measurement reliably can compare the amount of variation between any bioassay tests.
- h. **CS** = Check sample, *Bacillus* sample that is in the process of becoming validated as a RS.
- i. **RS** = Reference standard, a highly characterized *Bacillus* sample with known potency.
- j. **TS** = Test substance, *Bacillus* sample from a manufactured batch that requires comparison to the RS to determine the potency.
- k. **UTC** = Untreated control, treatments without *Bacillus* added to monitor bioassay contamination.
- l. **ITU** = International toxic units, international standard of measure for Bacterial Larvicides which consist of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* products.
- m. **ISS** = Initial stock suspension, solution created when *Bacillus* is initially added to stock bottles.
- n. **FSS** = Final stock suspension, solution created when ISS undergoes serial dilution
- o. **FTC (or C)** = Final test concentration, the six concentrations created by adding different amounts of FSS to the bioassay test cups.
- p. **High kill** = A replicate is considered high kill when the lowest mortality rate is greater than 50%

of the total number of insects.

- q. **Low kill** = A replicate is considered low kill when the highest mortality rate is lower than 50% of the total number of insects.
- r. **LC<sub>50</sub> Out of Range** = The LC<sub>50</sub> is out of range when the LC<sub>50</sub> value falls outside of the concentration range for that particular replicate.
- s. **LD<sub>50</sub> (or LC<sub>50</sub>)** = The lethal dose (or lethal concentration) of the pesticide at which 50% of the larvae are predicted to die.
- t. **Measurement Uncertainty (UM)** = a non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used.
- u. **Minitab® software** is statistical software that has various capabilities to analyze data. In Minitab, probit analysis is classified under reliability/survival rate category. Probit analyses qualify the survivorship data of the insect larvae used in the *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) bioassays.
- v. **Outlier** = An observation point that is statistically inconsistent with other observation points within a data set. There may be more than one outlier, if any, within a data set.
- w. **Potency** = The estimated LC<sub>50</sub> value of the pesticide reference standard divided by estimated LC<sub>50</sub> of the test substance (or check sample) and then multiplied by the potency of the reference standard.
- x. **Probit** = A statistical test with a binary response variable (dead or alive insect larvae) and one explanatory variable (six pesticide concentrations). This type of statistical model creates a regression line that predicts the likelihood of the outcome of interest at difference levels of the explanatory variable (in this case the rates of larval mortality by increasing pesticide concentrations). Results of a Probit analysis produce a LC<sub>50</sub> value that is used to estimate the potency of the *Bti* or *Bs* sample.
- y. **Raw Data** = Defined as any data to be entered into Microsoft Excel and then loaded into Minitab or other equivalent statistical software for Probit analysis to generate the final results of a bioassay.
- z. **Tween 80** = Polyoxyethylene-sorbitan mono-oleate, wetting agent

#### 4. Procedure

##### a. Equipment



- i. Metered dispensing pump
- ii. Refrigerator
- iii. Analytical balance
- iv. Shaker
- v. Bottle top dispenser
- vi. Graduated cylinders
- vii. Pipet aids
- viii. Desiccator jar
- ix. Transfer pipettes
- x. Sonicator
- xi. Sieve
- xii. Temperature/humidity controlled environmental chambers

##### b. Materials

- i. Deionized (DI) water
- ii. Tween-80 (polyoxyethylene-sorbitan mono-oleate)
- iii. Paper, wax-coated test cups
- iv. Paper, wax-coated dilution cups
- v. Mosquito *Aedes aegypti* larvae and/or *Culex quinquefasciatus* larvae

- vi. Wax paper
- vii. Disposable pipets
- viii. Glass bottles
- ix. Plastic bottles (autoclavable)
- x. Test trays
- xi. Approved disinfectant
- xii. Dishwashing liquid
- xiii. *Bacillus* reference standard (RS) and check sample (CS)
- xiv. Yeast extract (only when performing *Bacillus sphaericus* bioassays)

### c. Bioassay Procedure

- i. Set up activities indicated in below sections 2-8 may be completed the day prior to testing, however the UTC is set up the day of testing.
- ii. Set up the paper test cups on an appropriate size tray; the replications are described below. Each replicate consists of 6 final test concentrations (FTC) with three cups per concentration, and one untreated control (UTC) with three cups per test day. See Attachment 2 for bioassay scheme and replications layout.
  - a) RS requires three replicates per test day.
  - b) CS requires one replicate per test day.
    - a. In the event there is a shortage of insect larvae for testing, the CS may be eliminated
  - c) Due to variability of insect larvae, TS requires a minimum of two replicates per day (a minimum of four replicates over two test days). TS may not exceed four replicates per test day.
  - d) UTC contains three cups per test day.
- iii. RS/CS bottles, cups and dilution aides should utilize color differentiators for identification purposes (example):
  -  RS
  -  CS
- iv. Standardize bottle top dispenser.
- v. Use a 1-10 mL bottle top dispenser to dispense the correct amount of DI water into each cup (refer to Attachment 2 for dilution sequences).
- vi. Standardize water dispensing unit.
- vii. Dispense 90 mL of DI water into each test cup using water dispensing unit.
  - a) Dispense 100 mL of DI water into each UTC cup.
- viii. Add 100 mL of DI water or 0.2% Tween-80 solution to each RS, CS, or TS dilution bottle prior to weigh out. Dilution bottles for TS are labeled (hand-written or printed) with test substance lot number which may be abbreviated if desired and bioassay set up date. All Bti products must be added to 100mL of DI water, unless product is a GR (WHO monograph for granule) formulation in which 100mL of 0.2% Tween-80 solution is required- refer to Attachment 2 for preparation instructions. Once prepared, Tween solution expires after 24 hours.

*Note: All Bs products must be added to 100mL of 0.2% Tween-80, unless formulation is a SC formulation (WHO monograph for Suspension Concentrate) in which 100mL of DI water is required.*

### d. Infesting

- i. The environmental conditions of the insect colonies are controlled for consistency. Day-to-day consistency of the insect larvae is maintained by closely monitoring the

environmental chamber parameters and by limiting the handling time when manipulating the larvae outside of the chamber. The larvae should remain in the environmental chamber until the morning of testing and should be removed from the chamber at the exact same time (+/- 30 minutes) each day. The insect larvae begin to be infested into test cups within approximately 30 minutes of delivery to the bioassay lab. During this time, the insect larvae have no access to a food source for additional growth. If there is established consistency in both insect rearing and bioassay testing operations, the validity of test results should not be affected or impacted.

- ii. Insect larvae should be delivered each morning by insectary personnel.
- iii. Infest each UTC, RS, CS, and TS cup with 20 *Aedes* or *Culex* larvae beginning with the UTC.
  - a) The UTC is covered with wax paper and placed on designated cart immediately after infesting is complete.

***Very Important:***

- Larvae must be infested into the cups prior to setting up the dilutions or handling *Bacillus* samples to avoid contamination of the bioassay.
- Mosquito bioassay personnel should consult with Lab Management to determine whether bioassay should be performed if insect larvae do not meet requirements (for example, inconsistent in size, unhealthy appearance, etc.).
  - b) *Aedes* larvae are three days old, incubated at  $28 \pm 2^{\circ}\text{C}$ ,  $60\% \pm 15\%$  relative humidity, and are visually uniform in size.
  - c) *Culex* larvae are two days old, incubated at  $29.5 \pm 2^{\circ}\text{C}$ ,  $60\% \pm 15\%$  relative humidity, and are visually uniform in size.
    - a. If performing *Culex* (*Bs*) bioassay, 0.5mL of yeast solution must be dispensed into each test cup after infesting that will contain *Culex* larvae (refer to Attachment 2 for yeast solution preparation.). Yeast solution is prepared daily as needed.

**e. Sample Preparation/Testing Process**

- i. RS and CS are stored in a desiccator jar prior to use.
- ii. Obtain TS. TS requiring cold storage are set out at room temperature for approximately 1 hour prior to testing when testing requirements allow it.
- iii. RS, CS and TS Suspension Preparation
  - a) Target weights/dilutions are maintained by the test lab based on test history. To achieve valid mortality dose response, weigh the appropriate quantity of RS, CS and TS. The actual weight of any material weighed should be within proximity of the historical weight, unless additional information regarding expected potency is available from other sources.
  - b) Quantities weighed out may be adjusted if the estimated  $\text{LC}_{50}$  is too close to the highest or the lowest test concentration or outside of the range of concentrations tested.
    - a. Adjustments are based on several parameters, but not limited to historical data, reviewing kill patterns, etc.
  - c) Shake or stir (depends on type of formulation) each sample prior to weigh out to ensure sample is uniformly mixed.
  - d) Remove each substance from sample container by utilizing a spatula or transfer pipet. Place substance in weigh boat and use an analytical balance to weigh out each substance at its intended weight (mg) and add to designated dilution bottles. This makes the ISS.

- a. When weighing dry samples, if residue remains on weigh boat (for example when weighing WG formulations; WHO monograph for Water Dispersible Granules)- shake bottle, and using a transfer pipet, rinse residue utilizing water from dilution bottle.
- b. For all dry samples, wipe spatula with Kimwipe and/or Isopropyl Alcohol prior to moving to the next sample.

**Note: For DT formulations (WHO monograph for direct tablets) must first be crushed using mortar and pestle and ran through a 60-mesh sieve prior to weigh out.**

- e) Place bottles on shaker and shake dilutions bottles for approximately 20 minutes.
- f) *Culex* samples are placed in the sonicator for approximately 2-5 minutes.
- g) Prepare dilution cups for Final Stock Suspension (FSS). See Attachment 1 for example of dilution preparation.
- iv. Place FSS on the stir plate for approximately 10-15 seconds.
- v. Add the FSS for the RS, CS, or TS(s) to each cup. Refer to Attachment 1 for the amounts of FSS to be added to cups in order to achieve the FTC for each cup.
- vi. Once FTC are completed, cover the tray with an appropriate-sized wax paper. Make sure the wax paper covers all test cups and place on designated cart. Once all UTC, RS, CS, and TS trays have been covered and placed on cart, place the cart directly in the environmental chamber.
  - a) *Aedes (Bti)* bioassay tests must be incubated for 17-20 hours at  $28 \pm 2^\circ\text{C}$  and at  $55\% \pm 15\%$  relative humidity.
  - b) *Culex (Bpsh)* bioassay tests must be incubated for 42-45 hours at  $29.5 \pm 2^\circ\text{C}$  and at  $55\% \pm 15\%$  relative humidity.

## 5. Analysis

### a. Bioassay Test Reading

- i. Remove tests from the environmental chamber.
- ii. Begin by recording the number of dead insect larvae per cup (if any) for the UTC.
  - a) Count number of pupae beginning with UTC, including all RS, CS and TS. Use handheld counter if needed. Total number of pupae is recorded.
- iii. Next record the number of dead insect larvae per cup for the RS/CS trays and TS.
- iv. High kill/Low kill determination

Species	Concentration	Valid	High kill	Low kill
<i>Aedes</i>	C1	59	60	29
	C2	45	57	26
	C3	36	49	20
	C4	27	44	18
	C5	12	36	9
	C6	5	31	6
		<b>Lowest valid number before high kill</b>	<b>High kill</b>	<b>Highest valid number before low kill</b>
	30	31 and above	30	29 and below
Species	Concentration	Valid	High kill	Low kill
<i>Culex</i>	C1	51	60	29
	C2	42	59	29
	C3	34	52	17
	C4	18	48	15
	C5	10	33	7

	C6	6	31	9
	<b>Lowest valid number before high kill</b>	<b>High kill</b>	<b>Highest valid number before low kill</b>	<b>Low kill</b>
	30	31 and above	30	29 and below

- a) In the event a replicate is high or low kill, document high/low kill on the appropriate test record, along with any other appropriate descriptive information.
    - a. Along with valid data, high kill and low kill data will also need to be entered and run with the probit analysis program selected.
    - b. In rare occasions where either all insect larvae or no insect larvae were killed, this data will not be added to probit analysis.
  - v. Once all test reads are complete, place a sieve into the sink and pour the FTC cups (still containing insect larvae) through the sieve. Double bag the cups and discard.
  - vi. Strain insect larvae from sieve and into a container containing bleach and dish soap.
  - vii. Disinfect trays with 10% bleach solution followed by 70% IPA.
- b. Validity Criteria (prior to running probit analyses if applicable)**
- i. The entire test day is considered invalid and must be documented on the test packet and discarded if any of the following conditions are present:
    - a) The larval mortality of the UTC is greater than 15%.
    - b) More than 5% of insect larvae for the day have pupated, as this means the insect larvae are no longer ingesting the test substance.
    - c) All three RS trays are considered either high or low kill.
  - ii. The test day is considered valid if at least one replicate of the RS provides valid results.
  - iii. In the event any of the above data rejection occurrences are due to a laboratory error, the laboratory must conduct a Laboratory Investigation.
- c. Perform Probit analyses (Probit software is readily available. Minitab and Polo PC are examples)**
- i. The lethal concentration ratio is the average of all valid RS LC<sub>50</sub>s divided by the LC<sub>50</sub> of CS. This ratio indicates the relative potency of the two substances being compared. LC<sub>50</sub> is used to predict CS potency. Calculate Estimated CS and TS Potency using this formula:

$$\text{CS Potency} = \frac{\text{LC}_{50} \text{ average of RS1, RS2 and RS3}}{\text{LC}_{50} \text{ of check sample}} \times \text{RS potency}$$

- a) Calculate the CV of each TS utilizing the equation below.

$$\text{CV} = \frac{\text{standard deviation of the mean}}{\text{estimated mean test sample potency}} \times 100$$

- d. Validity Criteria (after running Probit analyses)**
- i. The results of a replication will be considered unacceptable if the estimated LC<sub>50</sub> value does not fall within its tested concentration range. If so, comment on the test package that

the LC<sub>50</sub> was outside of the concentration range along with any other appropriate descriptive information.

- a) The LC<sub>50</sub> of at least 1 RS replicate must be within range. Only RS replicates within range are used for potency calculations.
- b) A minimum of four TS replicates from at least two days for each TS is required.
- c) One of the following must also apply:
  - a. The 95% confidence interval is less than or equal to 15% of the mean (or % CV) when 4-9 valid reps have been obtained. Once 10 valid reps are reached the % CV is not considered as acceptance criteria; however, one may conduct additional reps if the CV is too high to be confident in the potency accuracy.
- ii. A total of 10 acceptable potency estimates have been obtained.

**e. Evaluating Potency Data for Outliers**

- i. A calculation utilizing the Modified Thompson Tau method will be used to confirm outlier(s). To be accepted as an outlier, the delta must meet or exceed the threshold value determined by the Modified Thompson Tau method.
  - a) Delta is the difference between the replicate's potency and the overall mean potency. The threshold value is the Modified Tau value multiplied by the Standard Deviation.
- ii. Look at the Tau\*Std and remove any potencies that indicate a Delta higher than the Tau\*Std (example below).

	Potencies	Delta	
Rep 1	22284	1878.375	
Rep 2	27898	7492.375	
Rep 3	21811	1405.375	
Rep 4	17876	2529.625	
Rep 5	17071	3334.625	
Rep 6	21875	1469.375	
Rep 7	17461	2944.625	
Rep 8	16969	3436.625	
Rep 9		20405.63	
Rep 10		20405.63	
Rep 11		20405.63	
Rep 12		20405.63	
Rep 13		20405.63	
Rep 14		20405.63	
Rep 15		20405.63	
Rep 16		20405.63	
Rep 17		20405.63	
Rep 18		20405.63	
Rep 19		20405.63	
Rep 20		20405.63	
Rep 21		20405.63	
Rep 22		20405.63	
Rep 23		20405.63	
Rep 24		20405.63	
Standard Deviation	3813.4733	Tau	1.7491
Average	20406	Tau*Std	6670.146
Coefficient of Variation	19 %	# of Reps	8

**f. Verifying/ Evaluating Data**

- i. All raw data and statistical calculations must be checked for accuracy and completion by a second person.



- a) Compare mortality totals to spreadsheet totals ensuring all numbers are accurate.
  - b) Verify weights and dilutions on the test packet match the spreadsheet.
  - c) Check all concentrations for each dilution.
  - d) Starting with the RS section, verify each replicate matches and that the LC<sub>50</sub>s are correct.
  - e) Check all LC<sub>50</sub>'s against the spreadsheet to ensure they fall within their designated concentration.
  - f) At times there can be 50% mortality, however the LC<sub>50</sub> may be out of range. Calculate the RS average and perform calculations of potencies of each TS.
  - g) Confirm the potencies are documented correctly on the test packet- this includes the replicate reads.
- ii. In the event any errors are found, correct errors and rerun analysis.

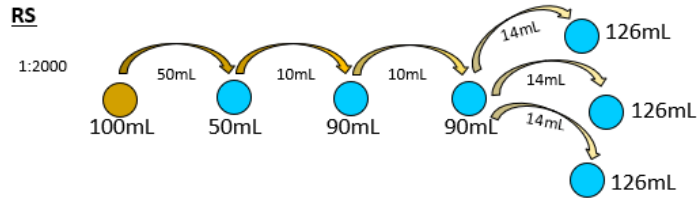
## 6. References

- a. Lacey, L. 1997. Bacteria: Laboratory Bioassay of Bacteria against Aquatic Insects with emphasis on Larvae of Mosquitoes and Black Flies. In Lacey, L. (Ed.). Manual of Techniques in Insect Pathology. Academic Press, Inc.
- b. Cymbala, J.M. 2011. Outliers. <https://www.mne.psu.edu/me345/Lectures/Outliers.pdf>. Pennsylvania State University
- c. Probit Analysis in Minitab. <http://support.minitab.com/en-us/minitab/17/topic-library/modeling-statistics/reliability/types-of-reliability-analyses/probit-analysis/>

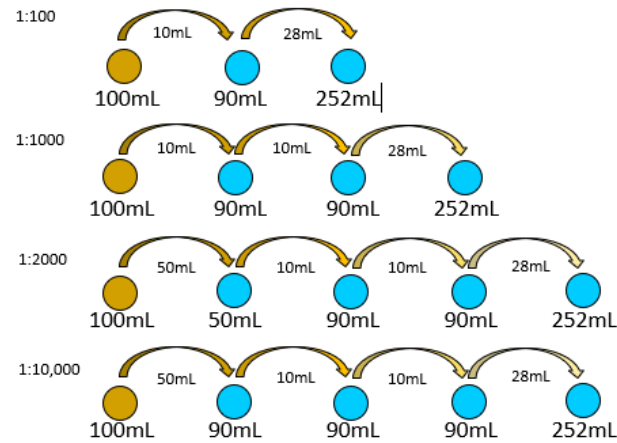
# Attachment 1

## Dilution Schemes

### *Aedes aegypti* (*Bacillus thuringiensis israelensis*)



#### **Test Substance**

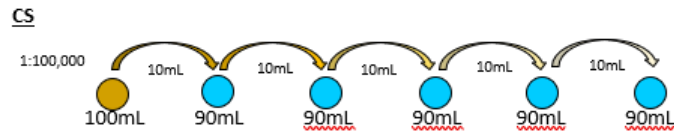
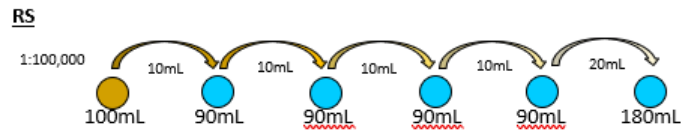


**Note:**

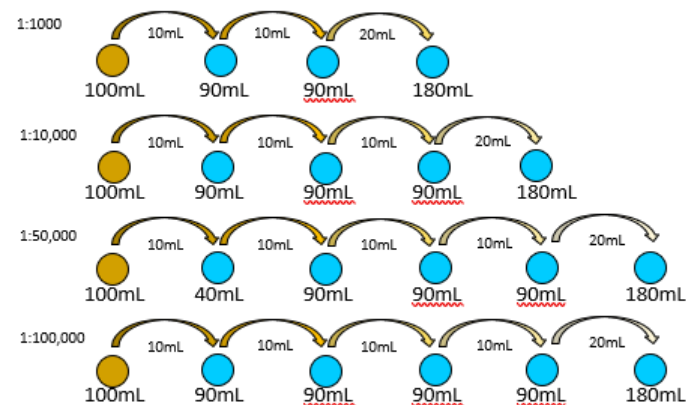
126 ml represents 1 replicate

252 ml represents 2 replicates

### *Culex quinquefasciatus* (*Bacillus sphaericus*)



#### **Test Substance**



Initial Stock Solution (ISS)

Wax-coated cup of DI Water (FSS)

**Note:**

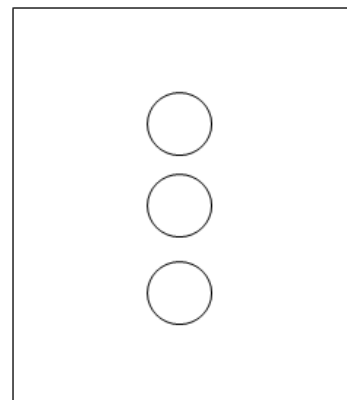
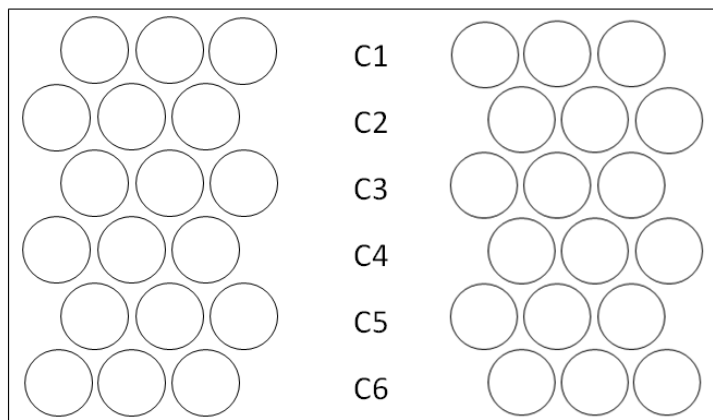
90 ml represents 1 replicate

## Attachment 2

### Bioassay Preparation Scheme, FTC Preparation, and Yeast and Tween Solutions Preparation

Each test tray will consist of 2 replicates. Cups will be set on trays as shown in the following figure.

Untreated Control (UTC) cups will be set up on designated trav.



#### FTC Preparation

	<i>Culex</i>	<i>Aedes</i>
C1	10mL FSS, 0mL DI Water	10mL FSS, 0mL DI Water
C2	5.5 FSS, 4.5mL DI Water	8.5mL FSS, 1.5mL DI Water
C3	3mL FSS, 7mL DI Water	7mL FSS, 3mL DI Water
C4	1.7mL FSS, 8.3mL DI Water	5.5mL FSS, 4.5mL DI Water
C5	0.9mL FSS, 9.1mL DI Water	4mL FSS, 6mL DI Water
C6	0.5mL FSS, 9.5mL DI Water	2.5mL FSS, 7.5mL DI Water

Yeast Extract for <i>Culex</i> Testing Only		
Yeast Extract (mg)	DI Water (mL)	Number of Trays
600	100	5
1200	200	10
1800	300	16
2400	400	22
3000	500	27

<b>0.2% Tween-80 Solution Preparation</b>		
<b>Tween-80 (mL)</b>	<b>DI Water (mL)</b>	<b>Total Volume (mL)</b>
1	499	500
2	998	1000
3	1497	1500
4	1996	2000

**Attachment 3**  
**Final Stock Solution Set-Up**

<b>Cup Set-Up</b>	
<b>Aedes</b>	<b>Culex</b>
<b>1:2000 RS, CS</b>	<b>1:100,000 RS, CS</b>
RS= 6 small cups CS= 4 small cups	RS= 4 small cups, 1 large cup CS= 5 small cups
<b>1:2000 Test Substance</b>	<b>1:100,000 Test Substance</b>
3 small cups, 1 large cup	4 small cups, 1 large cup
<b>1:1000 Test Substance</b>	<b>1:50,000 Test Substance</b>
2 small cups, 1 large cup	4 small cups, 1 large cup
<b>Adding DI Water to Cups</b>	
<b>1:2000</b>	<b>1:100,000</b>
1 @ 50ml	4 @ 90ml
2 @ 90ml	1 @ 90ml x 2 shots of water
1 @ 126ml x 2 shots of water	CS only 5 @ 90ml
<b>1:1000</b>	<b>1:50,000</b>
2 @ 90ml	1 @ 40ml
1 @ 126ml x 2 shots of water	3 @ 90ml
	1 @ 90ml x 2 shots of water
<b>Pipettes</b>	
<b>1:2000</b>	<b>1:100,000</b>
2- 10ml	4- 10ml
1- 25ml	1- 25ml
1- 50ml	CS only gets 5-10ml
<b>1:1000</b>	<b>1:50,000</b>
2- 10ml	4- 10ml
1- 25ml	1- 25ml