SOP: Bacterial Larvicide Bioassay

June 2023

Title	Bacterial Larvicide Bioassay (i.e. *Bacillus thuringiensis subsp. israelensis and Bacillus sphaericus*)
Document number	I2I-SOP-026
Version number	2
Date first published	13/07/21
Date last revised	

Prepared by

Name	Role	Institution
Annabel Murphy	Contributor	LSTM, I2I
Katherine Gleave	Contributor	LSTM, I2I
Peter DeChant	Author	Valent BioSciences LLC
Banugopan Kesavaraju	Author	Valent BioSciences LLC
Jennifer Burton	Author	Valent BioSciences LLC
Jason Clark	Author	Valent BioSciences LLC

Timeline

Version	Date	Reviewed by	Institution
1	12/07/23	Katherine Gleave	LSTM, I2I
2			

Version Control[1]

Version	Date	Updated by	Description of update(s)
2	04/07/23	Annabel Murphy	Updated: Format and structure under sub-headings and footnotes. Added

[1] Historical versions of SOPs can be found on the I2I website (https://innovationtoimpact.org/)

			glossary of terms and references. Created new tables.

Related documents

- I2I Best Practice SOP Library, 30 October 2020 (https://innovationtoimpact.org/)
- The Performance of Larval Insecticide Bioassays, LITSOP001.03
- Field Evaluation of microbial mosquito larvicide efficacy, I2I-SOP-027
- Insect Growth Regulator Larvicides, I2I-SOP-028
- Waste Disposal Procedure LITSOP008

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. Background

The generalized, internationally accepted method for determination of bacterial larvicide (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).

2.1. *Bacillus thuringiensis subsp. Israelensis*

- 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]

- 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 WHOPES in 2007.[3] 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).

2.2. *Bacillus sphaericus*

- 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 ABTS1743 material. [4]

- 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*

---

[2] 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.

[3] Ref 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.

[4] : 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[4]

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.

3. Materials and equipment

3.1. General equipment.

- Metered dispensing pump
- Refrigerator
- Analytical balance
- Shaker
- Bottle top dispenser

- Graduated cylinders
- Pipette aids
- Desiccator jar
- Transfer pipettes
- Sonicator
- Sieve
- Temperature/humidity controlled enviornmental chambers

3.2. General materials.

- Deionized (DI) water
- Tween-80 (polyoxyethylene-sorbitan mono-oleate)
- Paper, wax-coated test cups
- Paper, wax-coated dilution cups
- Mosquito *Aedes aegypti* larvae and/or *Culex quinquefasciatus* larvae
- Wax paper
- Disposable pipets
- Glass bottles
- Plastic bottles (autoclavable)
- Test trays
- Approved disinfectant
- Dishwashing liquid
- *Bacillus* reference standard (RS) and check sample (CS)

- Yeast extract (only when performing *Bacillus sphaericus* bioassays)

4. Procedure

4.2. Bioassay procedure

- Untreated control (UTC) is set up the day of testing.

- Set up the paper test cups on an appropriate size tray. 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 Figure 1 for bioassay scheme and replications layout.

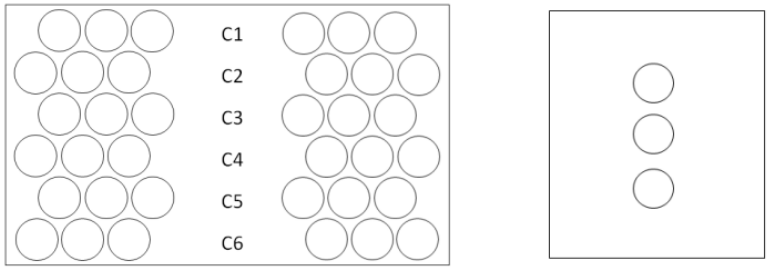

Figure 1: Cups set on trays with each test tray consisting of two replicates (left figure) and untreated controls (right figure)

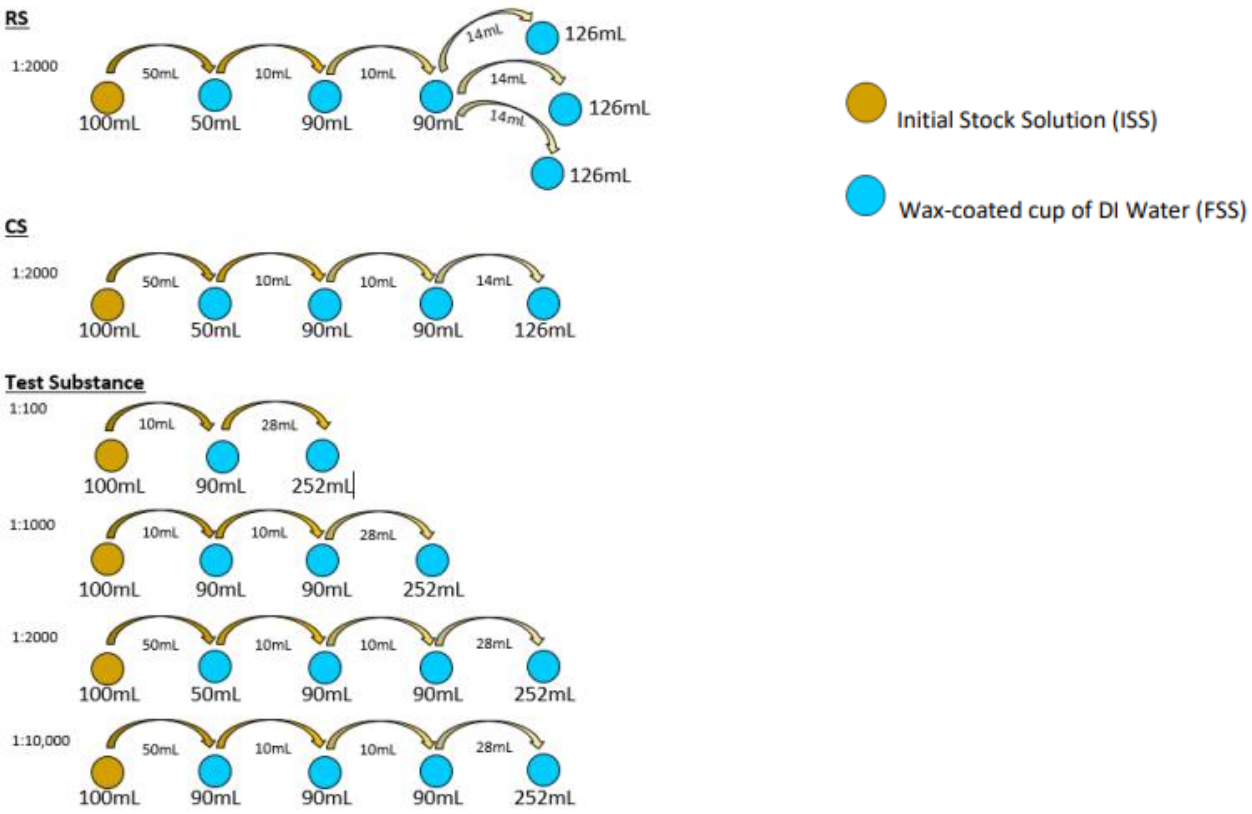

Figure 2: Dilution Schemes: Aedes aegypti (Bacillus thuringiensis israelensis. 126 mL represents 1 replicate. 252 mL represents 2 replicates.

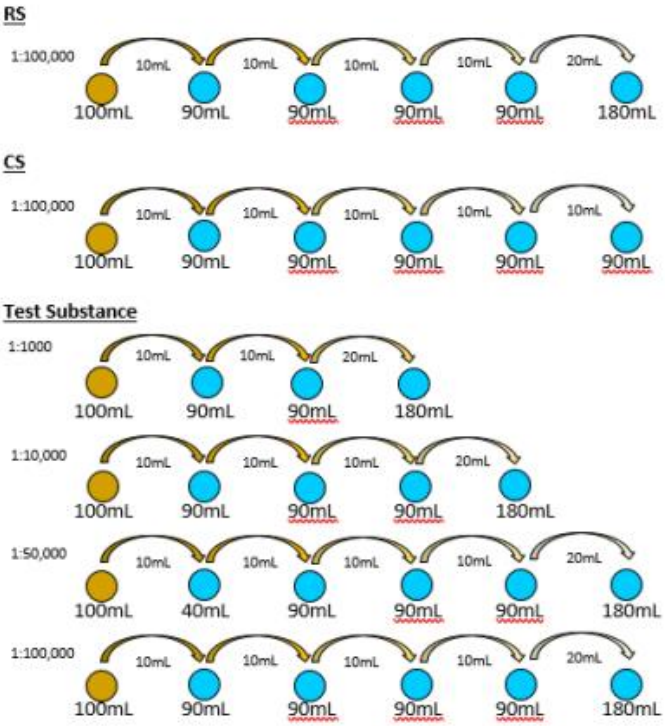

Figure 3: Dilution schemes: Culex quinquefasciatus (Bacillus sphaericus). 90mL represents 1 replicate.

- Reference Standard requires three replicates per test day.

- Control Standard requires one replicate per test day.

- Due to variability of insect larvae, the test substance (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.

- Untreated Control contains three cups per test day

- Reference Standard/Control Standard bottles, cups and dilution aides should utilize colour differentiators for identification purposes (Figure 4):

Figure 4: Colour differentiator example

- Standardize bottle top dispenser.

- Use a 1-10 mL bottle top dispenser to dispense the correct amount of DI water into each cup (refer to Figure 2 and 3 for dilution sequences).

- Standardize water dispensing unit.

- Dispense 90 mL of DI water into each test cup using water dispensing unit.

- Dispense 100 mL of DI water into each UTC cup.

- Add 100 mL of DI water or 0.2% Tween-80 solution to each RS, CS, or TS dilution bottle prior to weigh out. Tween solution preperation is shown in Table 1. 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 are added to 100mL of DI water. If it is a granule formulation 100mL of 0.2% Tween-80 solution is used. Once prepared, Tween solution expires after 24 hours.

Table 1: 0.2% Tween-80 Solution Preparation

Tween-80 (mL)	DI water (mL)	Total Volume (mL)
1	499	500
2	998	1000
3	1497	1500
4	1996	2000

4.3. Infesting

- Larvae must be infested into the cups prior to setting up the dilutions or handling Bacillus samples to avoid contamination of the bioassay.

- *Aedes* larvae are three days old, incubated at 28 ± 2°C, 60% ± 15% relative humidity, and are visually uniform in size. *Culex* larvae are two days old, incubated at 29.5 ± 2°C, 60% ± 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 Table 2 for yeast solution preparation.) Yeast solution is prepared daily as needed.

Table 2: Yeast solution preparation.

Yeast Extract for *Culex* 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

- 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 affected or impacted.

- Infest each UTC, RS, CS, and TS cup with 20 *Aedes* or *Culex* larvae beginning with the UTC.

- The UTC is covered with wax paper and placed on designated cart immediately after infesting is complete.

4.5. Sample Preparation/Testing Process

- RS and CS are stored in a desiccator jar prior to use.

- Obtain TS. TS requiring cold storage are set out at room temperature for approximately 1 hour prior to testing when testing requirements allow it.

- Prepare RS, CS and TS Suspension

    - 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.

    - Quantities weighed out may be adjusted if the estimated LC_{50} is too close to the highest or the lowest test concentration or outside of the

range of concentrations tested. Adjustments are based on several parameters, but not limited to historical data, reviewing kill patterns, etc.

- Shake or stir (depends on type of formulation) each sample prior to weigh out to ensure sample is uniformly mixed.

- 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 intial stock suspension (ISS).

- When weighing dry samples, if residue remains on weigh boat (for example when weighing Water Dispersible Granules formulations; - shake bottle, and using a transfer pipet, rinse residue utilizing water from dilution bottle.

- For all dry samples, wipe spatula with Kimwipe and/or Isopropyl Alcohol prior to moving to the next sample.

- For direct tablet formulations they must first be crushed using mortar and pestle and ran through a 60-mesh sieve prior to weigh out.

    - Place bottles on shaker and shake dilutions bottles for approximately 20 minutes.

    - *Culex* samples are placed in the sonicator for approximately 2-5 minutes.

    - Prepare dilution cups for Final Stock Suspension (FSS). See Attachment 1 for example of dilution preparation.

- Place FSS on the stir plate for approximately 10-15 seconds.

- Add the FSS for the RS, CS, or TS(s) to each cup. Refer to Table 3 for the amounts of FSS to be added to cups in order to achieve the final test concentration (FTC) for each cup.

Table 3: Final Test Concentration preparation

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

- 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.

    - *Aedes (Bti)* bioassay tests must be incubated for 17-20 hours at 28 ± 2°C and at 55% ± 15% relative humidity.

    - *Culex* bioassay tests must be incubated for 42-45 hours at 29.5 ± 2°C and at 55% ± 15% relative humidity.

4.6. Analysis: Bioassay Test Reading.

- Remove tests from the environmental chamber.

- Begin by recording the number of dead insect larvae per cup (if any) for the UTC.

- Count number of pupae beginning with UTC, including all RS, CS and TS. Use handheld counter if needed. Total number of pupae is recorded.

- Next record the number of dead insect larvae per cup for the RS/CS trays and TS.

- High kill/Low kill determination

Table 4: Validity criteria

Species	Concentration	Valid	High Kill	Low Kill
Aedes	C1	59	60	29
	C2	45	57	26

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

	Lowest valid number before high kill	High kill	Highest valid number before low kill	Low kill
	30	31 and above	30	29 and below

- 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.

- Strain insect larvae from sieve and into a container containing bleach and dish soap.

- Disinfect trays with 10% bleach solution followed by 70% Isopropyl alcohol.

- Refer to Waste Disposal Procedure LITSOP008 to correctly dispose of waste.

4.7. Validity Criteria (prior to running probit analyses if applicable)

- 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:

    - The larval mortality of the UTC is greater than 15%.

    - More than 5% of insect larvae for the day have pupated, as this means the insect larvae are no longer ingesting the test substance.

    - All three RS trays are considered either high or low kill.

- The test day is considered valid if at least one replicate of the RS provides valid results.

- In the event any of the above data rejection occurrences are due to a laboratory error, the laboratory must conduct a Laboratory Investigation.

4.8. Perform Probit analyses (Probit software is readily available. Minitab and Polo PC are examples)

- The lethal concentration ratio is the average of all valid RS LC_{50}s divided by the LC_{50} of CS. This ratio indicates the relative potency of the two substances being compared. LC_{50} is used to predict CS potency. Calculate Estimated CS and TS Potency using this formula:

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

- Calculate the CV[5]

4.9. Validity Criteria (after running Probit analyses)

- The results of a replication will be considered unacceptable if the estimated LC_{50} value does not fall within its tested concentration range. If so, comment on the test package that the LC_{50} was outside of the concentration range along with any other appropriate descriptive information.

    - The LC_{50} of at least 1 RS replicate must be within range. Only RS replicates within range are used for potency calculations.

[5] CV=Standard deviation of the mean/estimated mean test sample potency * 100

- A minimum of four TS replicates from at least two days for each TS is required.

- 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.

- A total of 10 acceptable potency estimates have been obtained.

4.10. Evaluating Potency Data for Outliers

- 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.

- 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.

- Look at the Tau*Std and remove any potencies that indicate a Delta higher than the Tau*Std (see Figure 5).

	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
Coefficent of Variation	19	%	# of Reps	8

*Figure 5: Tau*Std*

4.11.Verifying/Evaluating Data

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

    - Compare mortality totals to spreadsheet totals ensuring all numbers are accurate.

    - Verify weights and dilutions on the test packet match the spreadsheet.

- Check all concentrations for each dilution.

- Starting with the RS section, verify each replicate matches and that the LC_{50}s are correct.

- Check all LC_{50}'s against the spreadsheet to ensure they fall within their designated concentration.

- At times there can be 50% mortality, however the LC_{50} may be out of range. Calculate the RS average and perform calculations of potencies of each TS.

- Confirm the potencies are documented correctly on the test packet- this includes the replicate reads.

- In the event any errors are found, correct errors and rerun analysis.

5. Additional data collection

- Record time of testing.

- In the event there is a shortage of insect larvae for testing, the CS may be eliminated.

- 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.

- 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.

- Along with valid data, high kill and low kill data will also need to be entered and run with the probit analysis program selected.

- In rare occasions where either all insect larvae or no insect larvae were killed, this data will not be added to probit analysis.

6. Deviations from standard protocol

- All deviations from the standard protocol should be noted in the data collection sheets.

- All Bs products must be added to 100mL of 0.2% Tween-80, unless formulation is a suspension concentrate formulationin which 100mL of DI water is required.

- 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.).

7. Glossary of terms

Aedes	*Aedes aegypti*
Bs	*Bacillus sphaericus*
BL	Bacterial Larvicide
Bti	*Bacillus thuringiensis subsp. israelensis*

Culex	*Culex quinquefasciatus*
CV	Coefficient of variation
CS	Check sample (Bacillus sample that is in the process of becoming validated as a reference strain)
DI	Deionised water
FSS	Final stock suspension
FTC	Final test concentration
ITU	International Toxic Units
ISS	Initial stock suspension
ITU	International Toxic Units
LC_{50}	Lethal concentration 50%
L3	3^{rd} instar larvae
L4	4^{th} instar larvae
mg	milligram
mL	Milliliter

RS	Reference standard
SOP	Standard Operating Procedure
TS	Test substance
UTC	Untreated control
WHO	World Health Organisation
WHOPES	World Health Organisation pesticide evaluation scheme

8. References

Lacey, L. (1997). *Bacteria: Laboratory Bioassay of Bacteria against Aquatic Insects with emphasis on Larvae of Mosquitoes and Black Flies*. London, England: Academic Press.

(2023, July 11). *Probit Analysis in Minitab*. Retrieved from https://support.minitab.com/en-us/minitab/21/help-and-how-to/statistical-modeling/reliability/how-to/probit-analysis/perform-the-analysis/enter-your-data/