UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION
PREVENTION

April 29, 2020

MEMORANDUM

Subject: **Revised** Protocol Review for Reg. No. 94151PA7;
DP Barcode: 455973; Submission #: 1044629;

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Applicant: U.S. Food and Drug Administration (FDA), Center for Food Science and Applied Nutrition (CFSAN), Office of Regulatory Science (ORS)

Formulation from the Label: N/A

I. BACKGROUND

In response to the Agency protocol review, FDA provided the requiste clarification for full acceptance. The following review includes FDA's revisions, ATCC identification numbers, and EPA acceptance confirmation.

Product Description (as packaged, as applied): TBD

Submission type: Protocol Review

Currently registered efficacy claim(s): NA

Requested action(s): Requesting review/approval of protocols for industry to use for testing and claims.

Document(s) considered in this review:

- Revised proposed protocol

II. BRIEF DESCRIPTION OF THE PROTOCOL

Title: Efficacy protocol for reduction of foodborne bacteria in agriculture water for preharvest Due to lab capacity concerns, non-GLP (Good Laboratory Practice) data will be considered to support agricultural water submissions, provided that the study submission accurately represents how the study differs from the GLP standards in the 40 CFR 160.12 statement of non-compliance.

Purpose: The purpose of this assay is to determine the efficacy of a product to reduce foodborne bacteria in preharvest agricultural water using a modification of the AOAC Germicidal and Detergent Sanitizing Action of Disinfectants method.

Scope: 1) This test protocol is used to determine the effectiveness of a product for inactivating foodborne bacteria in preharvest agricultural water. The method of product application intended for the proposed protocol is not limited to liquid concentrates. It can be adapted to powders/granular chemistries as well.

2) For label amemdament, final label language should be developed in collaboration with EPA in the event that there are limitations with certain types of crops and/or other modifications.

3) Any modifications/deviations from the proposed protocol will require EPA review prior test initiation.

Test Substance Characterization: According to (40 CFR, Part 160, Subpart F [160.105]) test substance characterization as to identity, strength, purity, solubility and composition, as applicable, shall be documented before its use in this study. The stability of the test substance shall be determined prior to or concurrently with this study. Characterization and stability studies not performed following GLP regulations will be noted in the Good Laboratory Practice compliance statement.

Test System (Microorganisms):

Test Organism	ATCC numbers	Growth Medium
Shiga-toxin producing *E. coli*	43895 MP-9	Brain Heart Infusion (BHI)
Salmonella enterica CFSAN08857 CFSAN074573 CFSAN029938 CFSAN039243 CFSAN039249 CFSAN042339 CFSAN039113	ATCC® BAA-3138™ ATCC® BAA-3142™ ATCC® BAA-3140™ ATCC® BAA-3141™ ATCC® BAA-3137™ ATCC® BAA-3136™ ATCC® BAA-3139™	Brain Heart Infusion (BHI)
~~*Listeria monocytogenes*~~ ~~CFSAN034257~~ ~~CFSAN000763~~ ~~CFSAN002240~~ ~~CFSAN002285~~ ~~CFSAN006121~~	~~ATCC® BAA-3135™~~ ~~ATCC® BAA-3131™~~ ~~ATCC® BAA-3132™~~ ~~ATCC® BAA-3133™~~ ~~ATCC® BAA-3134™~~	~~Brain Heart Infusion (BHI)~~

Preparation of Test Organisms

1) Streak the test organisms on an agar plate of growth medium from a stock culture.
2) Incubate for 24±2 hours at 35-37 °C. Plates can be held at 4°C for a maximum of 7 days.
3) Inoculate a 10 μl loopful of colonies from the plate into one sterile 125 mL bottle containing 50 ml of Brain Heart Infusion Broth (BHIB).
4) Incubate the bottle for 24±2 hours at 35-37 °C to obtain the organism in the stationary growth phase.
5) Transfer 1 ml of culture to each sterile 1.5 ml Eppendorf tubes (6 tubes) without disturbing the culture.
6) Centrifuge tubes at 8,000 × g for 3 min.
7) Discard the supernatant. Resuspend the culture in 1 ml Phosphate Buffer Dilution Water (PBDW)
8) Standardize each culture to a common optical density, target: 1×10^{9} to 1×10^{10} CFU/mL (9-10 logs/mL). Additional PBDW may be added as necessary. The use of a spectrophotometer is recommended.
9) Combine equal volumes of the strains of each test organism. The result should be 4 sets of tubes (for the 2 pH levels and 2 temperature levels) for each test organism mixed culture. Each test organism mixed culture include:

(1) mixed culture of *Escherichia coli*, and (1) mixed culture of *Salmonella enterica*~~, and (1) mixed culture of *Listeria monocytogenes*~~. Each organism mixed culture set should be tested separately.

10) Add a 1.0 ml aliquot of mixed culture from step 9) midway between the center and edge of the flask by immersing the pipet tip into 98 ml of test agriculture water at pH 6.5 and at pH 8.4 in sterile 250-300 ml flask per test organism mixture. Avoid touching the side of the flask.
11) Swirl thoroughly to mix.
12) Equilibrate one set per pH (pH 6.5 and pH 8.4) at 12°C and the other at 32°C for at least 30 minutes.
13) Mixed cultures should be tested in triplicate per each pH and equilibrate temperature, with a total 12 flasks per each test organism.
14) The concentration of each mixed culture should be determined by plate count (Brain Heart Infusion Agar) after equilibration.

Preparation of Test Agriculture Water

Component	Amount Required	Finished Solution Parameter	Target Concentration
Sterile Deionized Water	1000 ml/L	Total Chlorine	<0.02 mg/L
PTI Arizona Test Dust	10 mg/L	Turbidity	≥100 NTU
Aldrich Humic Acid	10 mg/L	TOC	>10 mg/L
Sigma Sea Salts	1.6 g/L	TDS	1350-1650 mg/L
1 N HCl and/or 5N NaOH	As needed	pH	6.5 and 8.4

PTI Arizona Test Dust is added to clean, dry, sterile screw cap 1 L bottle. Approximately 500 mL of the total volume of deionized water is aseptically added to the container and mixed by shaking vigorously to achieve uniformity. The resultant suspension is added to the batch water and stirred vigorously. The turbidity of the water is determined using a Hach Laboratory Turbidimeter. The appropriate amount of Aldrich Humic Acid sodium salt is added to the bottle and approximately 500 mL of the test water is added to the bottle. The bottle is shaken vigorously for at least two minutes until the Humic Acid is fully hydrated and suspended. The resultant suspension is added to the batch water and stirred vigorously. The appropriate amount of Sigma Sea Salts is added directly to the batch water and stirred to dissolve. The pH of the final test water solution is adjusted to be 6.5 and 8.4, respectively. Two liters of the water per each pH value is prepared within one week prior to testing.

Preparation of Test Substance

1) Prepare the test substance at a concentration to meet the target concentration in a final volume of 100 ml.
2) Use the diluted test substance within 3 hours of preparation.

Test Substance Exposure

1) Add a 1.0 ml aliquot of test substance midway between the center and edge of the flask by immersing the pipet tip in the test organism/agriculture water mixture.
2) Avoid touching the side of the flask.
3) Swirl thoroughly to mix.
4) Allow for up to 5-minute contact time to elapse.

Test System Recovery

1) Following the contact period, transfer 1.0 ml of test substance + culture to 9 ml of neutralizer. Vortex to mix. This is the 10^{-1} dilution.
2) Prepare pour plates in quadruplicate using 4 (1) ml aliquots of neutralized material at the 10^{-1} dilution.
3) Prepare additional serial dilutions to 10^{-2}. Plate 0.1 ml aliquots of 10^{-1} & 10^{-2} dilutions in quadruplicate.
4) Incubate for 24-30 hours at 35-37°C. Visually examine for growth.

Study Controls

Sterility Controls

1) **Neutralizer sterility control**
   A representative sample of neutralizer (1.0 ml), per lot of neutralizer used in testing, will be plated onto the subculture agar medium as in the test. The plate will be incubated and visually examined. The acceptance criterion for this study control is a lack of growth.

2) **Test Substance Diluent Sterility Control**
   A sample of test substance diluent (1.0 mL), per lot used in testing, will be plated onto the subculture agar medium as in the test. The acceptance criterion for this study control is a lack of growth.

3) **Test Agriculture Water Sterility Control**
   One (1.0) mL of test agriculture water used in testing will be added to BHI agar plate, incubated and visually examined. The acceptance criterion for this study control is a lack of growth.

4) **PBDW Sterility Control**
   A representative sample of PBDW (1.0 mL), per lot used on the day of the test, will be plated onto the subculture agar medium as in the test. The acceptance criterion for this study control is a lack of growth.

Purity Control

Prepare a streak plate for each individual organism culture. Examine following to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.

Numbers Control using the Mixed Population

Transfer 98 mL of test agriculture water to a sterile 250-300 ml Erlenmeyer flask. Add 1.0 mL of 9-10 $\log_{10}$ CFU/mL mixed culture as in the test procedure. Swirl and equilibrate the flask for at least 30 minutes at each temperature as in the test procedure. One flask will be prepared to test each of the mixed cultures at each pH and temperature tested. Add 1.0 mL of PBDW instead of test substance in the protocol. Swirl thoroughly to mix.

Following contact time, transfer 1 mL of the contents to 9 mL of neutralizer. This represents the 10^{-1} dilution. Prepare ten-fold serial dilutions to 10^{-4}.

Prepare pour plates in quadruplicate using 4 (1) mL aliquots of 10^{-4} dilution.

Prepare pour plates in quadruplicate using 4 (0.1) mL aliquots of 10^{-4} dilution.

Incubate the plates as in the test.

The acceptance criterion for this control is a minimum value of 1×10^6 CFU/mL (6.0 $\log_{10}$) for each test organism mixture.

Neutralization confirmation tests

1) **Test Culture Titer (TCT)**
   Add 0.1 mL of diluted test organism to 10 ml of PBDW and vortex mix. Hold the mixture for a minimum of 2 minutes and spread plate or pour plate duplicate 0.1 mL aliquots as in the test. The acceptance criterion for this study control is growth.

2) **Neutralization Confirmation Control (NCC)**
   Prior to or concurrent with testing, perform the neutralization confirmation control for each of the three lots of test substance and each test organism mixture.

3) **Neutralization Confirmation Control Treatment (NCT)**
   Add 1.0 mL of test substance to 9 mL of neutralizer and vortex mix. Within approximately 30 seconds, add 0.1 mL of diluted test organism to the neutralized contents and vortex mix. Hold the mixture for a minimum of 2 minutes and spread plate or pour plate duplicate 0.1 mL aliquots as in the test. The acceptance criterion for this study control is growth 1 $\log_{10}$ of the test culture titer (TCT).

4) **Neutralizer Toxicity Treatment (NTT)**
   Add 0.1 mL of diluted test organism to 10 mL of neutralizer and vortex mix. Hold the mixture for a minimum of 2 minutes and spread plate or pour plate duplicate 0.1 mL aliquots as in the test. The acceptance criterion for this study control is growth within 1 $\log_{10}$ of the test culture titer (TCT).

Acceptance Criteria

The result should demonstrate a minimum of 3 $\log_{10}$ reduction of each of the test organisms as compared to the control count.

Calculations

Determine the CFU/mL for the test sample and numbers control using counts of 0-300.

$$\text{CFU/mL}=\frac{(average\ CFU\ for\ 10^{-x})+(average\ CUF\ for\ 10^{-y})+\cdots}{(10^{-x}+10^{-y}+\cdots)}$$

Where 10^{-x} and 10^{-y} are example dilutions plated.

All dilutions yielding counts between 0-300 will be used for the test subcultures.

The average $\log_{10}$ results of the triplicate test flasks will be averaged for each test organism/test substance set/temperature.

Log_{10} Reduction = Log_{10} (CFU/mL in the numbers control) – Average Log_{10} (CFU/mL in the test sample)

III. CONCLUSION AND COMMENTS

FDA has adequately addressed the comments from the Janary 28, 2020 protocol review. The Agency does not require any additional information.

Note for removing the Listeria monocytogenes panel in the protocol: This change is being made because pilot studies have found that sanitizer treatments that will likely be effective for *E. coli* and *Salmonella* may be different from those that are most effective for *L. monocytogenes*. This is likely due to the physical characteristics of *E. coli* and *Salmonella* being distinctly different from those of *L. monocytogenes*. In light of recent outbreaks of *E. coli* and *Salmonella* linked to produce, FDA and EPA agreed and decided to move forward with removing *L. monocytogenes* from the panel. We expect that doing so will facilitate the registration of antimicrobial treatments against *E. coli* and *Salmonella* in pre-harvest agricultural water, the availability of which will be a significant resource for farms to protect their crops against these pathogens. While we are removing *L. monocytogenes* from the protocol at this time, companies may opt to continue testing against *L. monocytogenes* for inclusion in their registration with EPA.