

**United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol**

SAM 612

Supplemental Assay Method for Bacterial Plate Count of *Erysipelothrix rhusiopathiae* Vaccines

Date: November 29, 2007

Number: SAM 612.02

Supersedes: STSAM0612.01, November 4, 1999

Standard Requirement: 9 CFR, Part 113.67

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Supplemental Assay Method for Bacterial Plate Count of *Erysipelothrix rhusiopathiae* Vaccines

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Supplemental Assay Method for Bacterial Plate Count of *Erysipelothrix rhusiopathiae* Vaccines

1. Introduction

This is a Supplemental Assay Method (SAM) for the analysis of *Erysipelothrix rhusiopathiae* vaccine, live culture, to determine the colony-forming units (CFU) in final container samples, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.67. This method uses 5% bovine blood agar and 1% peptone saline as a diluent.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

- 2.1.1 Vortex mixer
- 2.1.2 Colony counter
- 2.1.3 Sterile inoculum spreader
- 2.1.4 Disposable syringes and needles--appropriate sizes
- 2.1.5 Sterile disposable pipettes--appropriate sizes
- 2.1.6 Sterile screw-capped culture tubes, 20 x 150-mm
- 2.1.7 Pipetting aid
- 2.1.8 35°± 2°C incubator
- 2.1.9 Laminar flow biosafety cabinet
- 2.1.10 Gloves and lab coat
- 2.1.11 Sterile gauze pads, 4 x 4-inch
- 2.1.12 Test tube rack

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2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 1% Peptone-saline solution (**Appendix I**) - National Veterinary Services Laboratories (NVSL) Media #10138

2.2.2 Blood agar with 5% bovine blood (**Appendix II**) - NVSL Media #10006 or as stated in the Outline of Production (OP) from the biologics manufacturer

2.2.3 *E. rhusiopathiae* reference culture (American Type Culture Collection #19414)

2.2.4 70% ethyl alcohol

2.2.5 Sterile water in serum vials--volumes determined by referring to the biologics manufacturer's OP or as stated on the vaccine vial

3. Preparation for the Test

3.1 Personnel qualifications/training

The personnel performing this test must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures, policies, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn on the biosafety cabinet 1 hour before use.

3.2.2 Monitor the incubator, freezers, and coolers daily for temperature.

3.2.3 Label all plates with sample number or name, vial number, and dilution series. Label 3 plates per dilution series for each serial.

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3.3 Preparation of reagents/control procedures

3.3.1 Warm samples and reference culture to room temperature before rehydrating to the appropriate volume.

3.3.2 Prepare *E. rhusiopathiae* reference control samples according to manufacturer's instructions.

3.3.3 Negative and Positive Controls: Incubate 1 uninoculated plate of 5% bovine blood agar and 1 plate inoculated with sterile diluent along with the test sample plates as negative control plates. *E. rhusiopathiae* reference culture (positive control) is diluted the same as the test samples, but plated depending on the titer found in **Section 3.3.2**.

3.3.4 Store plates used for making counts at refrigerator temperature. Place plates to be used for counts in a $35^{\circ}\pm 2^{\circ}\text{C}$ incubator overnight prior to use or dry in a biosafety cabinet before use. At the time of use, plates are no more than 14 days old.

3.4 Preparation of the sample

Samples are *E. rhusiopathiae* and/or combination products containing this fraction.

4. Performance of the Test

4.1 Remove 2 vials of product to be tested and 1 vial of *E. rhusiopathiae* reference control sample from the freezer or cooler storage and allow to warm to room temperature.

4.2 Disinfect the cap with 70% ethyl alcohol. If needed, rehydrate the vials with the accompanying diluent or sterile water. Allow the contents of the vials to reconstitute for at least 5 minutes. Shake the vials by inversion until thoroughly mixed.

4.3 Prepare a tenfold dilution series of the product by setting up a rack of 20 x 150-mm screw-capped tubes and pipetting 9 mL of 1% peptone saline solution into each tube using a 10-mL pipette. Label the tubes 10^{-1} to 10^{-x} as needed.

4.4 Transfer 1 mL of the first sample from **Section 4.2** into the first tube of 1% peptone saline solution by using a pipette. Cap the tube and vortex. Continue the dilution series by using a pipette to transfer a 1-mL sample of this tube to the tube labeled 10^{-2} . Repeat this method using a sterile pipette for each transfer until the required number of serial tenfold dilutions, as determined from the release titer listed in the firm's OP, is attained.

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- 4.5** Repeat **Sections 4.3** through **4.4** with the second vial of product.
- 4.6** Deposit 0.1 mL of the sample from the last 3 dilution points of the dilution series for the product onto the surface of media in **Section 2.2.2** using a sterile pipette.
- 4.7** Use a sterile inoculum spreader to evenly distribute the inoculum over the surface of the agar medium.
- 4.8** Prepare 3 plates of media as in **Sections 4.6 and 4.7** from each of 3 reference control dilutions as determined from **Section 3.3.2** Use 1 uninoculated plate of media and 1 plate inoculated with sterile diluent as negative controls.
- 4.9** Invert all plates and incubate at $35^{\circ} \pm 2^{\circ}\text{C}$ for up to 72 hours. After incubation, count plates from each series that contains 30 to 300 CFU. Multiply the CFU by the dilution factor, average the CFU for the number of vials tested, and determine the CFU per dose by using the calculation listed below.

$$\frac{(\text{Average Count}) \times (\text{mL used to rehydrate})}{(\text{Dilution used}) \times (\text{mL plated}) \times (\text{Number of doses})} = \text{CFU/dose}$$

5. Interpretation of the Test Results

- 5.1** If on the initial test the CFU per dose is equal to or exceeds the required minimum as written in the firm's OP, the serial or subserial is satisfactory (SAT) for bacterial count without additional testing.
- 5.2** If on the initial test the CFU per dose is less than the required minimum as written in the firm's OP, the serial or subserial may be retested using 4 new vaccine samples, provided that if the retest (RT) is not done, the serial or subserial is unsatisfactory (UNSAT). Compare the firm's OP method to this SAM method when retesting the 4 vials. If on the RT, the average count of the 4 vaccine samples with the firm's OP method is less than the required minimum, the serial or subserial is UNSAT.
- 5.3** If on the RT, the average count of 4 vaccine vials is equal to or exceeds the required minimum, the serial is SAT.
- 5.4** If on the initial test the reference culture or positive control culture is not within the titer range determined in **Section 3.3.2**, but the serial being tested has a SAT result, the serial or subserial is a no test (NT) for bacterial count and the serial is released on the results of the firm's test. If the reference culture is not within its titer range and the serial being tested is below its minimum release titer, the serial is retested using 2 new vaccine samples. If on the initial test there is growth on the negative control plates, the serial or subserial is a NT for bacterial count without additional testing.

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6. Report of Test Results

Report the results of the test(s) as described by standard operating procedures.

7. References

Code of Federal Regulations, Title 9, Part 113.67, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- Amanda Byersdorfer has been added as a Contact.
- **2.1:** The Bunsen burner has been removed from the equipment list.
- **4.8:** The use of 1 plate inoculated with sterile diluent as a negative control to run sterility check on diluent used in testing has been added.
- **4.9:** The calculation to determine CFU/dose has been added.

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Appendices

Appendix I

NVSL Media #10138

Peptone solution 1% + 0.5% NaCl

Bacto peptone	10.0 g
NaCl	5.0 g
QH ₂ O	1000 mL

Autoclave for 20 minutes at 121°C.

Appendix II

NVSL Media #10006

Blood agar base with 5% bovine blood

Blood agar base (Difco)	40.0 g
H ₂ O	950 mL

Autoclave for 20 minutes at 121°C.

Cool to 47°C and add:

Defibrinated bovine blood	50 mL
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