United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 905

Supplemental Assay Method for Potency of Live Avirulent *Mannheimia haemolytica* Vaccine

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1. Introduction

This Supplemental Assay Method (SAM) establishes the titration method for the analysis of avirulent *Mannheimia* (formerly *Pasteurella*) *haemolytica* vaccine to determine the colony-forming units (CFU) in final container samples, per title 9, *Code of Federal Regulations* (9 CFR), part 113.68(c)(2). This method uses Trypticase Soy Broth (TSB) as a diluent and Trypticase Soy Agar (TSA) plates with 5% sheep blood for determining CFUs.

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 35°± 2°C incubator

2.1.4 Laminar-flow Class II biosafety cabinet (BSC)

2.2 Reagents/supplies

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

2.2.1 Trypticase Soy Broth (TSB) (**Appendix I**), National Centers for Animal Health (NCAH) Media #10404.

2.2.2 Trypticase Soy Agar with 5% sheep blood (**Appendix II**), NCAH Media #10218 or as stated in the Outline of Production (OP) from the biologics manufacturer

2.2.3 *M. haemolytica* reference culture (American Type Culture Collection (ATCC) #33396)

2.2.4 70% ethanol

2.2.5 Sterile water in serum vials

2.2.6 Inoculum spreader

2.2.7 Sterile syringes and needles

2.2.8 Sterile pipettes, individually packaged

2.2.9 Sterile screw-capped culture tubes

2.1.10 Lab coat and gloves

2.1.11 4 x 4-inch sterile gauze pads

2.1.12 Test tube rack

2.1.13 Sharps container

2.1.14 Pipetting aid

2.2.15 Micropipettors, 100-µL to 1.0-mL

2.2.16 Pipette tips, 100-µL to 1-mL

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel 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. Personnel must also have knowledge of safe operating procedures and 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 Operate all equipment and instrumentation according to the manufacturer's instructions and maintain according to standard operating procedures (SOPs).

3.2.2 Monitor temperature of incubators according to SOPs.

3.2.3 Turn on the BSC at least 30 minutes prior to testing.

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

3.3 Preparation of reagents/control procedures

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

3.3.2 *M. haemolytica* reference stock culture is prepared according to the manufacturer's instructions.

3.3.3 Negative and Positive Controls: Incubate 1 uninoculated plate of TSA with 5% sheep blood and 1 plate inoculated with sterile diluent with test sample plates as negative control plates. *M. haemolytica* 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. Plates to be used for counts are placed in a 35°± 2°C incubator overnight prior to use or allowed to dry in a BSC before use. At the time of use, plates are no more than 3 months old.

3.4 Preparation of the sample

Samples are *M. haemolytica* vaccines and/or combination products containing this fraction. Sterile purified water in serum vials in volumes specified on the product label or in the firm's OP are used for samples that are not accompanied by diluent.

4. Performance of the Test

4.1 Remove 2 vials (or the number of vials stated in the OP for testing) of product to be tested and 1 vial of *M. haemolytica* reference stock culture from the freezer or cooler storage and allow sample to warm to room temperature.

4.2 Disinfect the cap with 70% ethanol. Rehydrate the vials and allow the contents 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-cap tubes and pipetting 9.0 mL of TSB into each tube using a 10-mL pipette. Label the tubes 10^{-1} to 10^{-x} as needed.

4.4 Transfer 1.0 mL of sample from **Section 4.2** into the first tube of TSB by using a pipette or micropipettor with pipet tip. Cap the tube and vortex. The dilution series is continued by using a new pipette to transfer a 1.0 mL sample from 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 (refer to the firm's OP) is attained.

4.5 Deposit a 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 or micropipettor with pipet tip.

4.6 Use a sterile inoculum spreader to evenly distribute the inoculum over the surface of the agar medium.

4.6.1. The same inoculum spreader may be used on plates containing the same dilution. Change spreaders for each dilution plated.

4.6.2. Avoid spreading inoculum completely to the edges of the plate. Inoculum may pool at the edges, resulting in colony growth that is difficult to quantitate.

4.7 Repeat **Sections 4.4 through 4.6** with the second vial of product.

4.8 Prepare 3 plates of media as in **Sections 4.5 through 4.6** from each of 3 reference control dilutions as determined from **Section 3.3.2**.

4.9 Invert all plates and incubate at 35°± 2°C for up to 24 hours. After incubation, count plates from each series that contain 30 to 300 CFUs. Determine the mean CFU per dose for the number of vials tested using the calculation listed below.

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

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 double the amount of new vaccine samples used in the initial test, provided that if the retest is not done, the serial or subserial is unsatisfactory (UNSAT). Compare the firm's OP method to this SAM when retesting with the 4 new vaccine vials. If on the retest, the average count of the 4 new 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 retest with 4 new vaccine vials, the average using the firm's OP method count 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 without additional testing, and the product is released on the results of the firm's tests. If the reference culture is not within the titer and the serial being tested is below its minimum release titer, the serial is retested without bias using the same number of new vaccine samples as in the initial test. 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.

6. Record and Report of Test Results

Record and report results of the test(s) according to SOPs.

7. References

Title 9, *Code of Federal Regulations*, part 113.68(c)(2), U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .05

- The Bacteriology Section Leader has been updated.

Version .04

- *Pasteurella* in the title and throughout this document has been changed to reflect the name change to *Mannheimia*.
- The Bacteriology Section Leader has been updated.
- **1-5:** These Sections have been updated and rewritten for clarification.

Version .03

- **2-6:** These Sections have been updated to reflect current practices.

Version .02

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:

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- The document number has been changed from STSAM0905 to SAM 905.
- The Contact has been changed from Gerald Christianson to Sophia Campbell.
- **2.1:** The Bunsen burner has been removed from the list of equipment that is needed for the test.
- **3.2:** This section has been revised to reflect current practices.
- **4.:** This section has been rewritten for clarification.
- **4.8:** The use of 1 plate inoculated with sterile diluent for sterility check as a negative control plate has been added.
- **4.9:** The calculation for CFU/dose has been added.
- **Appendices:** Media storage conditions have been added.

Appendices

Appendix I

NCAH Media #10423

Trypticase Soy Broth (TSB) or Soybean Casein Digest Medium (SCDM)

Trypticase Soy Broth	30 g
QH_2O	1000 mL

Autoclave 20 minutes at 121°C. TSB and SCDM are 2 names for the same media formulation from different media companies. Store at 20°- 25°C for up to 3 months.

Appendix II

NCAH Media #10210

Trypticase Soy Agar (TSA) with 5% Sheep Blood

Trypticase Soy Agar	40 g
QH_2O	950 mL

Mix and autoclave 20 minutes at 121°C. Cool at 56°C in a waterbath. Add 50 mL defibrinated sheep blood. Store at 2°- 5°C for up to 3 months.