United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 912

Supplemental Assay Method for the Determination of Total Bacterial Count in Antibody Products

Date:	**February 21, 2023**
Number:	SAM 912.03
Supersedes:	SAM 912.02, January 12, 2017
Standard Requirement:	9 CFR 113.450(h)(2)(iv)
Contact:	Email: Methodsrequest.notification@usda.gov Phone: Center for Veterinary Biologics, 515-337-6100

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Supplemental Assay Method for the Determination of Total Bacterial Count in Antibody Products

Table of Contents

Supplemental Assay Method for the Determination of Total Bacterial Count in Antibody Products

1. Introduction

This Supplemental Assay Method (SAM) describes the test procedure used to determine the total bacterial count in dried and liquid antibody products for oral administration, per title 9, *Code of Federal Regulations* (9 CFR), part 113.450(h)(2)(iv). This test procedure uses Tryptone glucose extract agar (TGEA) to determine the colony-forming units (CFU) of contaminating bacteria.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Laminar-flow Class II biosafety cabinet (BSC)

2.1.2 Lab Armor® bead bath (set to 50°- 55°C)

2.1.3 Vortex mixer

2.1.4 30°- 35°C incubator

2.1.5 Analytical balance

2.1.6 Colony counter

2.2 Reagents/supplies

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

2.2.1 *Bacillus subtilis* (American Type Culture Collection (ATCC) #6633) or equivalent positive control culture

2.2.2 Tryptone glucose extract agar (TGEA) (**Appendix**), National Centers for Animal Health (NCAH) Media #50072 or as stated in the Outline of Production (OP) from the biologics manufacturer.

2.2.3 Sterile water

2.2.4 Sterile pipettes, individually packaged

2.2.5 Sterile petri dishes, 100 x 15-mm

2.2.6 Sterile culture tubes

2.2.7 Lab coat or sterile sleeves and gloves

2.2.8 Spatulas

2.2.9 Weigh boats

2.2.10 Sterile wide mouth snap cap specimen containers

2.2.11 70% ethanol

2.2.12 4 x 4-inch sterile gauze pads

2.2.13 Pipetting aid

2.2.14 Micropipettors, 100-µL to 1.0-mL

2.2.15 Pipette tips, 100-µL to 1.0-mL

2.2.16 Sterile syringes with needles

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 Turn on the BSC at the beginning of the work week and leave on all week.

3.2.3 Turn on the bead bath at least one day before use to allow the temperature to equilibrate.

3.2.4 Monitor temperature of incubators, freezers, coolers, and bead baths according to SOPs.

3.3 Preparation of reagents/control procedures

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

3.3.2 *Bacillus subtilis* reference stock culture is prepared according to the manufacturer's instructions and titrated to determine the CFU concentration. For each test session, inoculate two petri dishes with a volume containing approximately 100 CFU to serve as a positive control.

3.3.3 Negative/Technique Control: Inoculate one petri dish with 1 mL of the water that was used in the testing session. If no diluent is used in the testing session, use sterile water packaged in serum vials as the inoculum. Pour 15-20 mL of molten TGEA into each plate, swirl gently to mix and allow to solidify. Once the agar has hardened, invert the petri dish. Incubate this control plate with the serial test plates.

3.3.4 Preparation of the TGEA medium: On the day of the test, melt the TGEA in an autoclave for 30 minutes at 100°C. Place the bottles of melted media in the bead bath. Do not begin testing until the agar has cooled to at least 55°C. The temperature of the bead bath should be approximately 50°- 55°C when the agar is ready.

3.4 Preparation of the sample

3.4.1 At least ten containers of final product are required for total bacterial count purity testing.

3.4.2 Check the firm's OP, Section V, to obtain the maximum CFUs allowed in the product before determining the dilution scheme.

3.4.3 Samples are liquid or dried antibody products. When needed, sterile purified water in volumes specified on the product label or the diluent specified in the firm's OP is used for rehydrating samples.

3.4.4 Weigh samples of product on an analytical balance, if needed. Rehydrate samples of the product according to label or instructions from the firm's OP. Dissolve samples by stirring on a stir plate or shake with a vortex mixer. If sample does not readily go into solution after manipulating, allow the diluted sample to sit in a 30°- 35°C incubator for a minimum of 30 minutes.

3.4.5 Label 10 plates for each serial with the sample number or name and the container number.

4. Performance of the Test

4.1 Dress in a clean lab coat or sterile sleeves and gloves to perform purity testing.

4.2 Wipe down the interior surfaces of the BSC used for testing with 70% ethanol immediately prior to use and between testing each serial.

4.3 Place the necessary testing materials (micropipettors, pipet tips, 4 x 4-inch gauze squares, petri dishes, etc.) and the diluted sample into the BSC.

4.4 Mix the sample thoroughly. Dispense 1 mL of inoculum from one sample container into a petri dish.

4.5 Repeat **Section 4.4** for the other nine sample containers for this serial.

4.6 Dispense 15-20 mL of TGEA medium into each of the 10 dishes for this serial. Swirl gently to mix and then allow the agar to cool in the BSC. Once the agar has hardened, invert the petri dishes and place the dishes in a 30°- 35°C incubator for up to 48 hours.

4.7 Repeat **Sections 4.3** through **4.6** for the other serials of biologic to be tested.

4.8 Once all the test serials have been put on test, prepare the negative/technique controls for the testing session (see **Section 3.3.3**). Prepare negative controls using sterile water if no diluent was used in the testing session. Dispense 1 mL of water into a petri dish and pour 15-20 mL of TGEA into the plate. Swirl gently to mix and allow the agar to cool in the BSC. Once the agar has hardened, invert the petri dish.

4.9 Once the purity portion of the test has been completed, prepare the positive control organism in an area that is separate and apart from the clean area where the purity test was conducted (see **Section 3.3.2**).

4.9.1 Inoculate two petri dishes with approximately 100 CFUs of *B. subtilis*.

4.9.2 Dispense 15-20 mL of TGEA medium into each plate. Swirl gently to mix and then allow the agar to cool in the BSC. Once the agar has hardened, invert the petri dishes.

4.10 All plates are incubated at 30°- 35°C for up to 48 hours.

5. Interpretation of the Test Results

5.1 Criteria for a valid test:

5.1.1 There must be no growth in the negative/technique control and media control plates.

5.1.2 The positive control plates at the Center for Veterinary Biologics must contain an average count of 81-112 CFU.

Note: A range of approximately 100 CFU should be determined at each biologics manufacturer facility for each new positive control lot.

5.1.3 If these criteria are not met, the test is considered invalid or a no test (NT). Products may be reported and released with a NT result if there is no reason to suspect an unsatisfactory (UNSAT) sterility result for that product.

5.2 After incubation, all plates are examined. Plates that contain 30 to 300 bacterial colonies are counted with a colony counter. Determine the average count for the 10 plates counted.

5.3 Compare the average counts to the maximum CFU allowed in Section V of the firm's OP. If the average count is less than or equal to the maximum CFU allowed, the product is satisfactory (SAT) for the total bacterial count. If the average count is larger than the maximum CFU, the test is considered inconclusive (INC) so one retest (RT) to rule out faulty technique may be conducted. If a RT is not conducted within 21 days, the product is UNSAT by the first test.

5.4 If growth is observed on the negative or media control plate(s), or no growth is observed on the positive control plates, the test is a NT and may be repeated.

5.5 If a RT is ordered, double the number of samples used in the first test are required. If the average total count of the 20 plates from the RT is larger than the maximum CFU allowed, the serial is UNSAT.

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 113.450(h)(2)(iv), U.S. Government Printing Office, Washington, DC.

Standard methods for the examination of dairy products. 1992, 16th Edition, Robert T. Marshall, editor. American Public Health Association, Washington, D.C.

8. Summary of Revisions

Version .03

- The coversheet and the formatting have been updated.

Version .02

- Section 5.1.2 was rewritten for clarification.

Appendix

Tryptone Glucose Extract Agar (TGEA) – National Centers for Animal Health (NCAH) Media #50072

Tryptose Glucose Extract Agar	24.0 g
QH_20	1000.0 mL

Autoclave 20 minutes at 121°C. Store at 2°- 5°C for up to 3 months.