United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 928

Supplemental Assay Method for Detection of Extraneous Bacteria and Fungi in Live Bacterial Vaccines and Master Seed Bacteria Samples

Date:	**January 3, 2025**
Number:	SAM 928.08
Supersedes:	SAM 928.07, June 25, 2021
Standard Requirement:	9 CFR, Part 113.27(b) and (d)
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 Detection of Extraneous Bacteria and Fungi in Live Bacterial Vaccines and Master Seed Bacteria Samples

Table of Contents

Supplemental Assay Method for Detection of Extraneous Bacteria and Fungi in Live Bacterial Vaccines and Master Seed Bacteria Samples

1. Introduction

This Supplemental Assay Method (SAM) describes the test procedures used to detect viable extraneous bacteria and fungi in live bacterial vaccines and master seed bacteria (MSB) samples, as per title 9, *Code of Federal Regulations* (9 CFR), part 113.27(b) and (d). Extraneous viable bacteria or fungi are detected by comparison to an appropriate positive control by macroscopic evaluation on differential media and microscopic observation.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Microscope

2.1.2 Laminar-flow Class II biosafety cabinet (BSC)

2.1.3 30°- 35°C incubator

2.1.4 20°- 25°C incubator

2.2 Reagents/supplies

2.2.1 *Bacillus subtilis* (American Type Culture Collection (ATCC) #6633) or equivalent organism as specified in the current United States Pharmacopoeia (USP)

2.2.2 *Issatchenkia orientalis* (ATCC #6258) or equivalent organism as specified in the current USP

2.2.3 Trypticase Soy Broth (TSB) (National Centers for Animal Health (NCAH) Media #10423) (**Appendix I**)

2.2.4 Fluid Thioglycollate Medium (FTM), NCAH Media #10135 (**Appendix II**)

2.2.5 Trypticase Soy Agar (TSA), NCAH Media #10487 (**Appendix III**)

2.2.6 MacConkey Agar (MC), NCAH Media #10217 (**Appendix IV**)

2.2.7 Triple Sugar Iron Agar (TSIA), NCAH Media #10406 (**Appendix V**)

2.2.8 70% ethanol

2.2.9 Sterile water in serum vials

2.2.10 Lab coat or sterile sleeves and gloves

2.2.11 4 x 4-inch sterile gauze pads

2.2.12 Sterile syringes with needles

2.2.13 Vacutainer® needles

2.2.14 Sterile pipettes, individually packaged

2.2.15 Disposable inoculating loops

2.2.16 Microscope slides

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 one hour prior to testing.

3.2.3 Monitor the temperature of incubators, freezers, and coolers according to SOPs.

3.3 Preparation of reagents/control procedures

3.3.1 *Bacillus subtilis* stock culture is prepared according to the manufacturer's instructions and titrated to determine colony forming unit (CFU) concentration.

3.3.2 *Issatchenkia orientalis* stock culture is prepared according to the manufacturer's instructions and titrated to CFU concentration.

3.3.3 Positive control stock cultures are prepared according to the manufacturers' instructions and must be equivalent to the live bacterial product and/or the MSB being tested. The positive controls are used as a comparison to the organism(s) in the product.

3.3.4 Dilution of Preservative Screening (Eleventh Vessel Positive Control): For each serial of live bacterial vaccine tested, inoculate an additional container of medium for each incubation temperature with 0.2 mL of sample and approximately 100 CFU of the appropriate indicator organism (**Sections 2.2.1** and **2.2.2**). This control is used to confirm the ratio of inoculum to medium that will result in sufficient dilution of the product to prevent bacteriostatic and fungistatic activity according to 9 CFR 113.25(d).

3.3.5 Maximum Medium Volume Limits per Vessel: The maximum volume of medium per vessel used will not exceed 500 mL. Volumes greater than 500 mL will be divided evenly into two vessels and the volume of inoculum will be divided accordingly.

3.3.6 Negative Controls: Incubate 10 test vessels of each medium to confirm the sterility according to 9 CFR 113.25(c). Negative control test vessels are incubated with the serial test vessels at each temperature for the duration of the test.

3.4 Preparation of the samples

3.4.1 Samples to be tested are live bacterial vaccines and MSB. Ten vials of final product and a minimum of 4.0 mL MSB are required for sterility testing.

3.4.2 For products without accompanying diluent, rehydrate product with sterile purified water with the volume specified on the product label or in the Outline of Production (OP).

3.4.3 Follow Section V.A. of the OP for final product(s) to determine the volume of media per test vessel. Order a sufficient volume of media to accommodate the test vessels, positive controls, and negative controls.

4. Performance of the Test

4.1 Label the media test vessels to identify the serials and/or lots tested. Conduct all testing procedures in the BSC.

4.2 Disinfect the interior surfaces of the BSC with 70% ethanol. Disinfect the tops of the samples with a 4 x 4-inch gauze pad soaked in 70% ethanol.

4.3 Rehydrate each vial of product, if needed, using a syringe or a Vacutainer® needle with accompanying sterile diluent or sterile water.

4.4 Inoculate 0.2 mL rehydrated liquid or thawed vaccine or MSB into 10 vessels containing TSB and 10 vessels containing FTM. Swirl the vessel to distribute the product in the medium. Inoculate two TSA plates with one loopful of culture (approximately 10 µL) from each vial of product or MSB and streak for isolation; incubate one plate per incubation temperature. Smear one drop of culture on a glass slide and prepare for Gram staining and microscopic examination according to SOP.

4.5 Inoculate 0.2 mL of rehydrated or thawed vaccine into one additional vessel of TSB and FTM to serve as the 11th vessel positive controls (see **Section 3.3.4**). The sample may be obtained from any of the ten product containers or from an eleventh vial of product. Place the 11th vessels to the side and continue with the testing session.

Note: The 11th vessel positive controls are only used with live bacterial vaccines.

4.6 Conduct **Sections 4.3 through 4.5** for all vaccine serial(s) and **Sections 4.3 through 4.4** for MSB tested.

4.7 Conduct Sections **4.3 through 4.4** using the positive control(s) corresponding to the product being tested (see **Section 3.3.3**), except inoculate only two vessels per medium type.

4.8 Following the sterility portion of the test, prepare the 11th vessel control organisms in the BSC designated for this purpose (see **Sections 3.3.1** and **3.3.2**).

4.8.1 Inoculate approximately 100 CFU of the appropriate indicator organism into the vessels prepared in **Section 4.5** and swirl the vessel to distribute the organism in the medium.

4.8.2 Following inoculation of the 11th test vessels with the indicator organisms, inoculate two TSA plates per indicator organism with a representative volume of inoculum. These plates serve to demonstrate that the appropriate number of viable organisms were added to the test vessel.

4.9 Incubate test vessels containing FTM and half the inoculated TSA plates into a 30°- 35°C incubator. Incubate test vessels containing TSB and the other TSA plates into a 20°- 25°C incubator. Incubate the TSA plates up to 3 days. Incubate test vessels, negative controls, and positive controls for 14 days.

4.10 Following incubation, examine the TSA plates.

4.10.1 Look for abnormal growth and make slides of each colony type and Gram stain according to SOP. Compare colonial and cellular morphology to those of the positive control and record the results.

4.10.2 Count the colonies of 11^{th} vessel indicator organisms and the control plates.

4.11 Following incubation, examine the TSB and FTM cultures for atypical microbial growth. If growth of extraneous microorganisms cannot be reliably determined by visual examination, make a slide for microscopic examination and subculture for isolation.

4.11.1 Select three or more culture vessels per test serial per incubation temperature for further examination.

4.11.2 Smear one drop of culture on a glass slide and prepare for Gram staining and microscopic examination according to SOP.

4.11.3 Subculture the same test vessels onto MC, TSA, and TSIA slant. Incubate at the appropriate temperature for 3 days.

4.12 Compare colony morphology of the test sample to the positive control on MC and TSA cultures. Any differences may be further investigated by Gram staining and microscopic examination. Compare the TSIA reaction of the test sample to the positive control. Compare the cellular morphology of the test sample to those of the positive control.

5. Interpretation of the Test Results

5.1 The criteria for a valid test must be met or 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. Supervisor discretion is required for no test results of MSB.

5.1.1 There must be no growth in the Negative Control vessels.

5.1.2 The positive control produces expected growth.

5.1.3 For final products tested for dilution of preservative at the Center for Veterinary Biologics, the TSA plates containing *B. subtilis* must contain an average count of 81-112 CFU and the TSA plates containing *I. orientalis* must contain an average count of 76-124 CFU.

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

5.1.4 For final products tested for dilution of preservative, growth of the indicator organism must be observed in the 11th vessel positive control. If there is a lack of indicator organism growth in the 11th vessel positive control(s) in a final product, the serial will be evaluated utilizing a complete dilution of preservative study according to 9 CFR 113.25(d). If it is found that the volume of media listed in the OP for sterility testing permits interference in the dilution of preservative test, the test will be considered UNSAT and the sterility test will be reported as NT.

5.2 Master Seed Bacteria (MSB)

5.2.1 If extraneous growth is detected in any test vessel of the initial test for MSB, one retest (RT) may be conducted using new unopened vials of MSB. If extraneous growth is found in any test vessel of the RT, the MSB is UNSAT.

5.2.2 MSB that exhibit no extraneous growth are considered satisfactory (SAT).

5.3 Live Bacterial Vaccines

5.3.1 If extraneous growth is detected in 2 or 3 of the 20 test vessels of the initial test, one RT may be conducted using 20 new unopened samples. Conduct the RT in the same manner as **Sections 4.1 through 4.13** and omit the 11th vessel test, unless necessary. If a RT is not conducted within 21 days, the product is UNSAT by the results determined in the initial test.

5.3.2 If no extraneous growth is detected in 19 or 20 test vessels of the initial test, or 39 or 40 test vessels of the RT, the serial is considered SAT.

5.3.3 If extraneous growth is found in 4 or more of the 20 test vessels of the initial test, or 2 or more of the 40 test vessels in the RT, the serial is UNSAT.

5.4 If a MSB or live bacterial vaccine is found UNSAT, acquire a 3-4 mL sample of the contaminated culture, label the tube with sample identification, and freeze at -65°C or lower. The contaminant will also be identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

6. Record and Report of Test Results

Record and report test results as described by SOPs.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.27(b) and (d), U.S. Government Printing Office, Washington, DC.

7.2 The U.S. Pharmacopoeia, Mack Publishing Co., Eaton, PA, Vol. 21, 1985, pp 1151-1160.

7.3 Kurtzman, C. P., C. J. Robnett, and E. Basehoar-Powers. 2008. Phylogenetic relationships among species of Pichia, Issatchenkia and Williopsis determined from multigene sequence analysis, and the proposal of Barnettozyma genera novel, Lindnera genera novel and Wickerhamomyces genera novel. FEMS Yeast Res 8:939-54.

7.4 Center for Veterinary Biologics Notice 12-21.

8. Summary of Revisions

Version .08

- Updated Cover page to new format

Version .07

- Updated Sections 3 through 5.

Version .06

- The contact information, the Bacteriology Section Leader, and CVB-PEL Director have been updated.
- Updated **Sections 4.9** and **5.1**-**5.3**.
- **Appendix III-V:** Updated media storage limits.

Version .05

- Reformatted **Section 5** to clarify the test interpretation for MSB and live bacterial vaccines

- Clarified interpretation of test results for consistency.

Version .04

- Revised **Section 4.4** to include gram staining the sample directly from the vial.

Version .03

- Revised to include dilution of preservative requirements cited in CVB Notice 12-21.
- **Sections 2-6** have been updated to reflect current practices.
- **7.3:** Reference added for name change of *Candida krusei* to *Issatchenkia orientalis*.

Version .02

- The document number has been changed from STSAM0928 to SAM 928.
- The Contact has been changed from Dolores Strum to Sophia Campbell and Alaina Ingebritson.
- **1:** Information regarding use of this SAM for Master Seed Bacteria has been added.
- **2.1:** The Bunsen burner has been removed from the list of equipment that is needed for the test.
- **2.1.5:** The class of biosafety cabinet to be used has been added.
- **3.1:** Personnel qualifications have been clarified.
- **3.3.3/3.3.4:** These sections have been rewritten for clarification.
- **3.4.2:** This section has been rewritten for clarification.
- **3.4.3:** This section has been added to indicate that the volume of media cited in the Outline of Production is used for the testing.
- **4:** This section has been revised to clarify the procedures currently used.
- **5:** This section has been revised to clarify the interpretation of the test results.

- **Appendices:** Media storage conditions have been added.

Appendices

Appendix I

NCAH Media #10423

Trypticase Soy Broth (TSB)

Trypticase Soy Broth	30 g
QH_2O	1000 mL

Autoclave 20 minutes at 121°C. Store at 20°- 25°C for up to 3 months.

Appendix II

NCAH Media #10135

Fluid Thioglycollate Medium (BBL)

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 mL

Mix and heat to boiling. Autoclave 20 minutes at 121°C. Store at 20°- 25°C for up to 3 months.

Appendix III

NCAH Media #10487

Trypticase Soy Agar (TSA)

Trypticase Soy Agar (BBL)	40 g
QH_2O	1000 mL

Autoclave 20 minutes at 121°C. Store at 2°- 5°C for up to 6 months.

Appendix IV

NCAH Media #10217

MacConkey Agar

MacConkey Agar (BBL)	50 g
QH_2O	1000 mL

Autoclave 20 minutes at 121°C. Store at 2°- 5°C for up to 6 months.

Appendix V

NCAH Media #10406

Triple Sugar Iron Agar

Triple Sugar Iron Agar Slants (TSIA)	65 g
QH_2O	1000 mL

Autoclave 15 minutes at 121°C. Slant tubes at a 20° angle. Store at 2°- 5°C for up to 6 months.