United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 906

Supplemental Assay Method for Sterility Testing of Preparations Other Than Live Vaccines

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Table of Contents

1. Introduction

2. Materials

    2.1 Equipment/instrumentation
    2.2 Reagents/supplies

3. Preparation for the Test

    3.1 Personnel qualifications/training
    3.2 Preparation of equipment/instrumentation
    3.3 Preparation of reagents/control procedures
    3.4 Preparation of the samples

4. Performance of the Test

5. Examination of the Test Vessels

6. Interpretation of the Test Results

7. Record and Report of Test Results

8. References

9. Summary of Revisions

Appendices

Supplemental Assay Method for Sterility Testing of Preparations Other Than Live Vaccines

1. Introduction

This Supplemental Assay Method (SAM) describes the test procedure used to detect viable bacteria and fungi in all biological products other than live vaccines, per title 9, *Code of Federal Regulations* (9 CFR), part 113.26. In the presence of these contaminating extraneous agents, the medium will be rendered turbid by macroscopic examination.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 30°- 35°C incubator

2.1.2 20°- 25°C incubator

2.1.3 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 *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 Soybean Casein Digest Medium (SCDM) or 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 Fluid Thioglycollate Medium with Beef (FTM w/Bf), NCAH Media #10227 (**Appendix III**)

2.2.6 Trypticase Soy Agar (TSA) plates, NCAH Media #10487 (**Appendix IV**)

2.2.7 Glassware: tubes and flasks containing test media

2.2.8 Sterile water in serum vials

2.2.9 Lab coat or sterile sleeves and gloves

2.2.10 70% ethanol

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

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 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 Dilution of Preservative Screening (Eleventh Vessel Positive Control): For each serial tested, inoculate an additional container of media for each incubation temperture with 1.0 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).

3.3.4 Maximum Medium Volume Limits per Vessel: The maximum volume of media per vessel used will not exceed 500 mL as these large volumes are hazardous when removing from the autoclave. Volumes greater than 500 mL will be divided evenly into two vessels and the volume of inoculum will be divided accordingly.

3.3.5 Negative Control: Include 5 uninoculated test vessels of each media used in the testing session to confirm the sterility of the media batch according to 9 CFR 113.25(c). Incubate the representative test vessels at each incubation temperature for 14 days.

3.4 Preparation of the samples

3.4.1 Follow Section V.A of the Outline of Production (OP) for final product(s) to determine the type and/or volume of media that should be aliquoted into each vessel. The type of media used for testing is based on the product description. This information is compiled in **Appendix V**. Master Cell Stock (MCS), primary cells, and ingredients of animal origin are tested with 40 mL of medium at each temperature unless otherwise directed. Order a sufficient volume of media to accommodate the test vessels, positive controls, negative controls, and extra vessels for potential subcultures.

3.4.2 For products without accompanying diluent, order sterile purified water in serum vials in volumes specified on the product label or in the OP.

Note: Most nonlive products are liquid; rehydration is only occasionally required.

3.4.3 Ten vials of final product and a minimum of 20 mL MCS, primary cells, and ingredients of of animal origin are required for sterility testing.

4. Performance of the Test

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

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

4.3 Place the necessary testing materials (syringes, Vacutainer® needles, 4 x 4-inch gauze squares, etc.), test media, and the product to be tested in the BSC.

4.4 Swab the top of each container of product, diluent, and water with a gauze pad soaked in 70% ethanol.

4.5 If necessary, rehydrate each lyophilized sample container of product using a syringe and needle or Vacutainer® needle. Use the diluent provided with the product or sterile water. For completed product, reconstitute to the minimum volume as indicated on the product label or Section VI of the OP.

4.6 Withdraw product from each container with a new sterile syringe and needle. Dispense a 1.0 mL aliquot from each product vial into one vessel of each media type used for testing (one vessel per incubation temperature). If the recoverable volume from an individual container of product is less than 2.0 mL, inoculate ½ the sample volume into each test vessel. For MCS, primary cells, and ingredients of animal origin, dispense 1.0 mL alquots into each test vessel. Swirl each test vessel after inoculating with sample to distribute the product in the media.

4.7 Inoculate one additional vessel of each type of media with 1.0 mL of product to serve as the 11th vessel positive controls (see **Section 3.3.3**). The product sample for these control vessels may be obtained from any of the previously used containers or from an eleventh vial of product. Set the 11th vessels to the side and continue with the testing session.

Note: The 11th test vessel is not conducted with MCS, primary cells, and ingredients of animal origin.

4.8 When all of the test vessels have been inoculated with product, set-up the negative control for the testing session (see **Section 3.3.5**).

4.9 Once the sterility portion of the test has been completed, prepare the positive control organisms in an area that is separate and apart from the clean area where the sterility test was conducted (see **Sections 3.3.1** and **3.3.2**). The type of organism to be used for each product is specified in **Appendix V**.

4.9.1 Inoculate approximately 100 CFU of the appropriate indicator organism into the vessels prepared in **Section 4.7** and swirl the vessel to distribute the organism in the medium.

4.9.2 After the 11th test vessels have been inoculated with the indicator organisms, inoculate one TSA plate per indicator organism with a representative

volume of inoculum. This plate count serves to demonstrate that the appropriate number of viable organisms were added to the test vessel.

4.10 Incubate the test vessels and the representative control at the appropriate incubating temperature. Incubate all vessels for 14 days. Incubate the TSA plates containing the indicator organisms for a maximum of 7 days.

4.11 Wipe down the interior of the BSC and counter tops with 70% ethanol at the end of the testing session. Discard biological samples and contaminated materials according to SOPs.

5. Examination of the Test Vessels

5.1 Examine all test vessels for cloudiness or turbidity at least once during the testing period. Observe bacterins, bacterin-toxoids and clostridial toxoids on days 7 to 11; MCS, primary cells, and ingredients of animal origin on days 3-7; and observe all samples on test on day 14.

5.2 Determine if the cause of cloudiness or turbidity is due to microbial growth by subculturing the contents of the test vessel by aseptically transfering 1.0 mL from the test vessel in question to 20 - 25 mL of fresh test media. Swirl the vessel to mix the inoculum into the media and incubate at the appropriate temperature for a minimum of 3 days. In addition, subculture the test vessel culture onto two plates of blood agar and TSA and streak for isolation. Incubate one plate of each at both incubation temperatures for 3 days.

5.3 On day 14, vessels without growth are considered negative.

5.4 By day 14, any vessels with bacterial or fungal growth confirmed by growth in the subcultures are considered positive for extraneous growth. The contaminant will also be identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS).

6. Interpretation of the Test Results

6.1 Criteria for a valid test:

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

6.1.2 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.

6.1.3 For final products tested for dilution of preservative, growth of the indicator organism must be observed in the 11th vessel positive control.

6.1.4 If 6.1.3 criteria are not met, the test is considered invalid/no test and the final product will be retested without bias. If 6.1.3 criteria are not met upon retest, the product will undergo SAM 903 based testing to determine ultimate compliance with 9 CFR 113.25(d) requirements as described in Veterinary Services Memo (VSM) 800.120. The sterility result will be reported as "no test" if 9 CFR 113.25(d) dilution of preservative testing is ultimately determined to be "unsatisfactory." Refer to Section 6.5, SAM 903, and VSM 800.120 for further information.

6.2 If no growth is found in any test vessel, the serial is satisfactory (SAT).

6.3 If growth is observed in any test vessel and confirmed by subculture, the test may be repeated using 20 unopened final container samples.

6.4 If extraneous growth is found in any test vessel of the final test, the serial is unsatisfactory (UNSAT).

6.5
A prelicense serial that fails the initial and retest using the 11th vessel screen and subsequently fails SAM 903 based testing has the interim designation of "no test" for all sterility testing and "unsatisfactory for dilution of preservative." Generally, no conditional use or full product license will be issued until 9 CFR 113.25(d) compliance is resolved. The biologics manufacturer will be informed of the interim results by the assigned CVB reviewer and given a designated time to respond with a plan to address 9 CFR 113.25(d) requirements for the product. If the CVB does not receive an acceptable response from the biologics manufacturer within the defined time-period, in consultation with the assigned reviewer, the CVB laboratory will report out the final prelicense serial test result as "no test" for all sterility testing and "unsatisfactory" for dilution of preservative. Regulatory flexibility may be exercised for specific instances involving emergency use authorization for a product.

A serial from a licensed product that fails initial and retest using the 11th vessel screen and subsequently fails SAM 903 based testing will be reported out with the testing result of "no test" for all sterility testing and "unsatisfactory" for dilution of preservative.

Consult Veterinary Services Memo (VSM) 800.120 for more information on 9 CFR 113.25(d) compliance and dilution of preservative studies.

7. Record and Report of Test Results

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

8. References

8.1 Title 9, *Code of Federal Regulations*, parts 113.25 and 113.26, U.S. Government Printing Office, Washington, DC.

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

8.3 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.

8.4 SAM 903, Supplemental Assay Method for Testing for Preservative Interference with Sterility Tests

8.5 VSM 800.120, Dilution of Preservative Testing as a Part of Sterility Testing of Veterinary Biologics.

9. Summary of Revisions

Version .06

- Updated the cover page, Section 6.1.4, and 6.5.
- Added Sections 8.4 and 8.5

Version .05

- Updated cover page.
- Updated Sections 2 through 6.

Version .04

- The Bacteriology Section Leader has been updated.
- Updated Sections 2.2, 3.3, and 4.
- Deleted Appendix V (Blood Agar base with 5% bovine blood – NVSL Media #10006).

Version .03

- Revised to include dilution of preservative requirements cited in CVB Notice 09-02.
- The Contact information has been updated.
- **Sections 3.3, 4, 5, and 6** have been updated to reflect current practices.
- **8.3:** Reference added for name change of *Candida krusei* to *Issatchenkia orientalis*.
- **Appendices:** Updated media storage limits to be in compliance with 9 CFR 113.25(b).

Version .02

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail and the following changes were made to the document:

- The Contact has been changed from Gerald Christianson to Sophia Campbell and Amanda Byersdorfer.
- **2.1** The Bunsen burner has been removed from the list of equipment that is needed for the test.
- **3.3.3:** This section has been added to describe the 11th vessel positive control.
- **3.3.4:** This section has been added to clarifiy media vessel volume limits used in testing. A note on the safety hazard when removing volumes > 500 mL from the autoclave has also been added.
- **3.4.3** The practice of looking up the volume of test media needed for each serial to be tested on the dilution of preservative computer disk using the notebook program has been removed.
- **6.3:** Clarification on how to interpret the 11th vessel positive control results has been added, along with information on follow-up testing.
- Brain heart infusion agar and Trypticase soy agar with 5% sheep's blood have been removed from this outline and replaced with blood agar base with 5% bovine blood plates and trypticase soy agar plates.
- Media storage information has been added in the Appendices.

Appendix I

Trypticase Soy Broth (TSB) or Soybean Casein Digest Medium (SCDM) - NCAH Media #10423

Trypticase Soy Broth	30 g
QH_2O	1000 mL

Autoclave 20 minutes at 121°C. Store at 20°- 25°C for up to 90 days.

TSB and SCDM are synonymous mediums.

Appendix II

Fluid Thioglycollate Medium (BBL) – NVSL Media #10135

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 90 days.

Appendix III

Fluid Thioglycollate with Beef Extract – NVSL Media #10227

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 mL

Heat and add:

0.5% Beef Extract (DIFCO)	5 g

Bring to a boil and dispense. Autoclave 20 minutes at 121°C. Store at 20°- 25°C for up to 90 days.

Appendix IV

Trypticase Soy Agar (TSA) – NVSL Media #10487

Trypticase Soy Agar	40 g
QH_2O	1000 mL

Boil to dissolve and autoclave for 15 minutes at 121°- 125°C. Store at 2°- 7°C for up to 90 days.

Appendix V

Media, incubation conditions, and control organisms to be used with each class of biological product

Product Description		
Killed products without merthiolate or clostridial components	FTM 30°-35°C	*Bacillus subtilis*
	SCDM 20°-25°C	*Issatchenkia orientalis*
Killed products with merthiolate but no clostridial components	FTM 30°-35°C	*Bacillus subtilis*
	FTM 20°-25°C	*Issatchenkia orientalis*
Killed products with clostridial components but no merthiolate	FTM w/Bf 30°-35°C	*Clostridium chauvoei** and *Bacillus subtilis***
	SCDM 20°-25°C	*Issatchenkia orientalis*
Killed products with clostridial components and merthiolate	FTM w/Bf 30°-35°C	*Clostridium chauvoei** and *Bacillus subtilis***
	FTM 20°-25°C	*Issatchenkia orientalis*
Plasma bags without merthiolate	FTM 30°-35°C	*Bacillus subtilis*
	SCDM 20°-25°C	*Issatchenkia orientalis*
Plasma bags with merthiolate	FTM 30°-35°C	*Bacillus subtilis*
	FTM 20°-25°C	*Issatchenkia orientalis*
Program disease products without merthiolate	FTM 30°-35°C	*Bacillus subtilis*
	SCDM 20°-25°C	*Issatchenkia orientalis*
Program disease products with merthiolate	FTM 30°-35°C	*Bacillus subtilis*
	FTM 20°-25°C	*Issatchenkia orientalis*

*9 CFR 113.25(b) testing.
**11th Vessel testing.