United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 903

Supplemental Assay Method for Testing for Preservative Interference with Sterility Tests

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Contact: Sophia G. Campbell, (515) 337-7489

Approvals: /s/Larry R. Ludemann Date: 06Jan16
Larry R. Ludemann, Section Leader
Bacteriology

/s/Paul J. Hauer Date: 11Jan16
Paul J. Hauer, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

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

Entered into CVB Quality Management System
by: /s/Linda S. Snavely 11Jan16
Linda S. Snavely Date
Quality Management Program Assistant

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1. Introduction

This Supplemental Assay Method (SAM) describes the test procedure used to determine the ratio of inoculum to medium for sterility testing that will result in a sufficient dilution of the product to prevent bacteriostatic and fungistatic activity, per title 9, *Code of Federal Regulations* (9 CFR), part 113.25(d).

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 30º- 35ºC incubator

2.1.2 20º- 25ºC incubator

2.1.3 Laminar-flow Class II biosafety cabinet (BSC)

2.1.4 Waterbath

2.2 Reagents/supplies

Equivalent reagents or supplies may be substitued 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 *Clostridium chauvoei* spores or equivalent organism as specified in the current USP

2.2.4 Brain-Heart Infusion Agar (BHIA), National Centers for Animal Health (NCAH) Media #10204 (**Appendix I**)

2.2.5 Trypticase Soy Agar (TSA) or Soybean-Casein Digest Agar (SCDA), NCAH Media #10487 (**Appendix II**)

2.2.6 Soybean Casein Digest Medium (SCDM) or Trypticase Soy Broth (TSB) NCAH Media #10423 (**Appendix III**)

2.2.7 Fluid Thioglycollate Medium (FTM), NCAH Media #10135 (**Appendix IV**)

2.2.8 Fluid Thioglycollate Medium with Beef (FTM w/Bf), NCAH Media #10227 (**Appendix V**)

2.2.9 Sterile pipettes, individually packaged

2.2.10 Lab coat or sterile sleeves and gloves

2.2.11 70% ethanol

2.2.12 4 x 4-inch sterile gauze pads

2.2.13 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 least one hour prior to testing.

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

3.3 Preparation of reagents

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

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

3.3.3 *C. chauvoei* stock culture is prepared according to the manufacturer's instructions and titrated to determine the proper use-dilution.

3.3.4 To test for preservative interference of aerobic bacterins and killed virus biologics, use *B. subtilis* with FTM at 30º- 35ºC and *I. orientalis* with TSB or FTM at 20º- 25ºC. For anaerobic bacterins, use *C. chauvoei* with FTM w/Bf at 30º-35ºC and *I. orientalis* with TSB or FTM at 20º- 25ºC. For parenteral live viral products, use *B. subtilis* with TSB at 30º- 35ºC and *I. orientalis* with TSB at 20º- 25ºC. For chicken embryo origin (CEO) and certain poultry vaccines used for mass inoculation via drinking water, use *B. subtilis* with BHIA at 30º- 35ºC and *I. orientalis* with BHIA at 20º- 25ºC.

4. Performance of the Test

4.1 Broth media

4.1.1 For each biological product being tested, prepare 10 test vessels of each media specified for both incubation temperatures. In addition to the media for each test sample, prepare 10 test vessels of the appropriate media for each indicator organism to serve as positive controls (**Section 3.3.4**).

4.1.2 For each sample being tested, inoculate each of the 20 prepared vessels with 1 mL or 0.2 mL of the sample; the volume of inoculum depends on the requirement of the sterility test (9 CFR 113.26 or 113.27).

4.1.3 Following inoculation with the test sample(s), add approximately 100 CFU of the appropriate indicator organism to the sample test vessels. Swirl the vessels to distribute the sample and the organism in the medium.

4.1.4 Inoculate each of the 20 prepared vessels for the positive controls with approximately 100 CFU of the appropriate indicator organism. Swirl the vessels to distribute the organism in the medium.

4.1.5 Incubate and observe for growth all test vessels inoculated with *B. subtilis* or *C. chauvoei* at 30º- 35ºC for 7 days and test vessels inoculated with *I. orientalis* at 20º- 25ºC for 14 days.

4.2 Agar media

4.2.1 Agar pour-plates are used for sterility testing CEO and other poultry vaccines administered via drinking water. For each biological product being tested, prepare 10 sterile petri dishes for each incubation temperature. In addition to the the test sample, prepare 10 petri dishes for each indicator organism (**Section 3.3.4**).

4.2.2 For each test sample, inoculate all 20 petri dishes with 10 bird doses of product. Following distribution of the test sample, add approximately 100 CFU of the appropriate indicator organism to 10 sample petri dishes. Following preparation of the test sample petri dishes, distribute approximately 100 CFU of of indicator organism into 10 petri dishes each.

4.2.3 Following distribution of the test sample(s) and indicator organisms into the petri dishes, gently pour the appropriate volume of melted BHIA (cooled to about 50º-60°C) into each petri dish. Gently swirl the petri dishes to mix the inoculum into the agar. Allow the agar to cool and harden for up to 2 hours before inverting for incubation.

4.2.4 Incubate the plates inoculated with *B. subtilis* at 30º- 35ºC for 7 days and the plates inoculated with *I. orientalis* at 20º- 25ºC for 14 days.

5. Interpretation of the Test Results

5.1 There is no interference in the broth test if the vessels containing biological product exhibit equivalent growth of the indicator organism compared to the positive control vessels containing no biological product.

5.2 There is no interference in the agar test if the plates containing biological product have an average CFU within 20% of the positive control plates containing no biological product.

5.3 If interference is demonstrated by reduced growth of the indicator organisms in the presence of the biological product, the test procedure must be repeated using a range of greater media volumes to establish the appropriate volume of media required for sterilty testing.

6. Record and Report of Test Results

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

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.25(d), U.S. Government Printing Office, Washington, DC.

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

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.

8. Summary of Revisions

Version .04

- The Bacteriology Section Leader has been updated.
- Updated **Sections 1**, **2**, and **4.1**.
- **Appendices:** Updated media storage limits to be in compliance with 9 CFR 113.25(b).

Version .03

- **Sections 1-4** have been updated to reflect current practices.
- **7.3:** Reference added for name change of *Candida krusei* to *Issatchenkia orientalis*.

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:

- The document number has been changed from STSAM0903 to SAM 903.
- The Contact has been changed from Gerald Christanson to Sophia Campbell.
- **2.1:** The equipment/instrumentation list has been updated to include sterile test vessels and to clarify that the Biosafety Cabinet is to be Class II.
- **Appendices:** Media storage conditions have been added.

Appendices

Appendix I

NCAH Media #10204

Brain Heart Infusion Agar (BHIA)

Brain Heart Infusion Agar	52.0 g
QH_2O	1000 mL

Autoclave 20 minutes at 121ºC. Store at 2°- 5°C for up to 90 days.

Appendix II

NCAH Media #10487

Trypticase Soy Agar (TSA) or Soybean-Casein Digest Agar (SCDA)

Trypticase Soy Agar	40.0 g
QH_2O	1000 mL

Autoclave 20 minutes at 121ºC. Store at 2°- 5°C for up to 90 days.

Appendix III

NCAH Media #10423

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

Trypticase Soy Broth	30.0 g
QH_2O	1000 mL

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

TSB and SCDM are synonymous mediums.

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Appendix IV

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 90 days.

Appendix V

NCAH Media #10227

Fluid Thioglycollate with Beef Extract

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 mL

Heat and add:

0.5% Beef Extract (Difco)	5.0 g

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