United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 908

Supplemental Assay Method for Sterility Testing of Live Viral Vaccines and Master Seed Virus Samples

Date: **September 3, 2015**

Number: SAM 908.05

Supersedes: SAM 908.04, November 13, 2013

Standard Requirement: 9 CFR Part 113.27(a) and (c)

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

Approvals: /s/Larry R. Ludemann Date: 11Sep15
Larry R. Ludemann, Section Leader
Bacteriology

/s/Byron E. Rippke Date: 15Sep15
Byron E. Rippke, Acting 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 15Sep15
Linda S. Snavely Date
Quality Management Program Assistant

Table of Contents

Supplemental Assay Method for Sterility Testing of Live Viral Vaccines and Master Seed Virus Samples

1. Introduction

This Supplemental Assay Method (SAM) describes the test procedure used to detect viable bacteria and fungi in all live viral vaccines and Master Seed Virus samples, per title 9, *Code of Federal Regulations* (9 CFR), parts 113.27(a) and (c). In the presence of 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* (formerly *Candida krusei*, 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 Trypticase Soy Agar (TSA) plates, NCAH Media #10487 (**Appendix II**)

2.2.5 Glassware: tubes and flasks containing test media

2.2.6 Sterile water in serum vials

2.2.7 Lab coat or sterile sleeves and gloves

2.2.8 70% ethanol

2.2.9 Sterile gauze pads, 4 x 4-inch

2.2.10 Sterile syringes with needles

2.2.11 Vacutainer® needles

2.2.12 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, 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* (formerly *Candida krusei*) 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 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 conducted 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 Technique Control: Inoculate each of 20 test vessels of media with 0.5 mL of sterile water packaged in serum vials. Use the same lot of water and syringes used with the tested biologicals. Technique control test vessels are incubated with the serial test vessels for 14 days at each incubation temperature.

3.3.6 Media Control: Include 20 uninoculated test vessels of media to confirm the sterility of the media batch according to 9 CFR 113.25(c). Incubate 10 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 volume of media that should be aliquoted into each vessel. Master Seed Virus (MSV) samples are tested with a minimum of 120 mL of TSB at 30°- 35°C and 40 mL of TSB at 20°- 25°C. 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.

3.4.3 Ten vials of final product and a minimum of 4.5 mL of MSV 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 and between testing each serial or lot.

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 gauze soaked in 70% ethanol.

4.5 For live viral vaccines, rehydrate 10 vials of each lyophilized serial with water or accompanying diluent using a syringe and needle or Vacutainer® needle. Rehydrate products for mass inoculation with sterile water at a rate of 30 mL per 1000 doses or as specified on the product label or OP. If necessary, MSV is rehydrated with TSB (SCDM).

4.6 Withdraw product from each sample cantainer with a new sterile syringe and needle. Dispense a 0.2 mL aliquot from each vial of the rehydrated liquid or thawed frozen liquid product into two test vessels (one tube per incubation temperature). Dispense 0.2 mL of rehydrated or thawed MSV samples among the 20 test vessels. Swirl each test vessel after inoculating with sample to distribute the product in the media.

4.7 Inoculate two additional vessels of media with 0.2 mL of product to serve as the 11^{th} 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 11^{th} vial of product. Set the 11^{th} vessels to the side and continue with the testing.

Note: The 11^{th} vessel positive control is not conducted with MSV.

4.8 When all of the test vessels have been inoculated with product, prepare the negative controls for the testing session (see **Sections 3.3.5** and **3.3.6**). It is acceptable to prepare a single set of negative controls for multiple products being tested concurrently.

4.9 Once the sterility portion of the test has been completed, prepare the postive 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**).

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 11^{th} 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 controls 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 vessel medium for cloudiness or tubidity at least once during days 7 to 11 of the incubation period and on day 14.

5.1.1 Determine if the cause of cloudiness or turbidity is due to microbial growth by conducting a Gram stain on an aliquot of the culture.

5.1.2 If growth in a vessel cannot be reliably determined by Gram stain, subculture the contents of the test vessel by aseptically transfering 1.0 mL from the test vessel in question to 40.0 mL of fresh test media. Swirl the vessel to mix the inoculum into the media and incubate 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.

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

5.3 By day 14, any vessels with bacterial or fungal growth confirmed by Gram stain and/or growth in the subcultures are considered positive for extraneous growth.

6. Interpretation of the Test Results

6.1 Criteria for a valid test:

6.1.1 There must be no growth in any of the Technique Control or Media Control vessels.

6.1.2 For final products tested for the dilution of preservative, the TSA plates containing the indicator organisms must contain approximately 100 cfu/plate.

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

6.1.4 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 sterility result for that product.

6.2 For final product:

6.2.1 If extraneous growth is found in 2 or 3 of the 20 test vessels of the initial test, one retest (RT) may be conducted using 20 unopened final container samples.

6.2.2 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 satisfactory (SAT).

6.2.3 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 of the RT, the serial is unsatisfactory (UNSAT).

6.2.4 If there is no growth in the 11th vessel positive control(s), there will be a 3-week delay before the test results are reported *(Center for Veterinary Biologics Notice 09-02,* http://www.aphis.usda.gov/animal_health/vet_biologics/publications/notice_09_02.pdf) while a dilution of preservative study will be conducted on the serial according to 9 CFR 113.25(d). If the volume of media listed in the OP for sterility testing of the serial indicates interference in the 9 CFR 113.25(d) test, this testing will be reported as UNSAT and the sterility test result will be reported as a NT.

6.3 For MSV:

6.3.1 If extraneous growth is observed in any test vessel of the initial test, one RT may be conducted using a new sample of MSV.

6.3.2 If extraneous growth is found in any test vessel of the final test, the lot of MSV is UNSAT.

7. Report of Test Results

Record and report the test results as described by SOPs.

8. References

8.1 Title 9, *Code of Federal Regulations*, parts 113.25 and 113.27, 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.

9. Summary of Revisions

Version .05

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

Version .04

- Clarified interpretation of test results in Section 6.2**.**

Version .03

- Revised to include dilution of preservative requirements cited in CVB Notice 09-02.
- The Contact phone number has been updated.
- **Sections 3, 4, 5, and 6:** These sections 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 Dolores Strum to Sophia Campbell.
- **2.1** The Bunsen burner has been removed from the list of equipment that is needed for the test.
- **3.3.2:** This section has been added to describe the 11th vessel positive control.
- **3.3.6:** This section has been added to clarify media volume limits per vessel used in testing. Also added a note on the safety hazard when removing volumes > 500 mL from the autoclave.

- **3.4.3** Determination of the volume of test media needed for each serial to be tested on the dilution of preservative computer file using Lotus Approach 97 has been removed.

- **4.1** Disinfection of the vials of the serials of biologic to be tested with 0.05% Germ Warfare using a Clean-Pal or equivalent has been removed.

- **4.8** Instead of "dehydrating" the "desiccated" vials, the instruction has changed to "rehydrating" the "lyophilized" vials.

- **5.1:** The section has been rewritten to indicate that tubes chosen for subculturing are at the technican's discretion rather than randomly.

- **5.4**: Clarification on how to interpret the 11th vessel positive control results has been added, along with information on follow-up testing.

- **5.6**: Additional information regarding contaminant labeling has been added.

- Media storage information has been added to the Appendix.

Version .01

The information contained in this document was previously available as a protocol (STPRO0270.01 dated September 9, 1996). The document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

Appendix I

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

Note: TSB and SCDM are synonymous mediums.

Appendix II

Trypticase Soy Agar (TSA), NCAH 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.