United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 909

Supplemental Assay Method for Sterility Testing of Live Viral Vaccines of Chicken Embryo Origin Recommended for Nonparenteral Injection

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1. Introduction

This Supplemental Assay Method (SAM) describes the test procedure used to detect viable bacteria and fungi in live viral vaccines and master seeds (MS) of chicken embryo origin recommended for nonparenteral injection, per title 9, *Code of Federal Regulations* (9 CFR), part 113.27(e). This test procedure uses Brain Heart Infusion Agar (BHIA) to determine the colony-forming units (CFUs) of contaminating bacteria and fungi.

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)

2.1.4 Lab Armor® bead bath (set to 55°- 60°C)

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

2.2.4 Sterile water in serum vials, refer to **Appendix II**

2.2.5 Penicillinase Concentrate, 10,000,000 Kinetic (Kersey) units/mL (BBL catalog number 211898)

2.2.6 Petri dishes, 100 x 15-mm or 150 x 15-mm

2.2.7 Glassware: sterile Pyrex bottles, 500-mL with screw tops containing test media

2.2.8 Lab coat or sterile sleeves and gloves

2.2.9 70% ethanol

2.2.10 4 x 4-inch sterile gauze pads

2.2.11 Sterile syringes with needles

2.2.12 Vacutainer® needles

2.2.13 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 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 *Bacillus subtilis* stock culture is prepared according to the manufacturer's instructions and titrated to determine the colony forming unit (CFU) concentration. For each test session, inoculate two petri dishes with a volume containing approximately 100 CFU to serve as a positive control. This control is used to confirm that a sufficient number of viable organisms were added to the 11th Vessel Positive Controls.

Note: Incubate plates inoculated with *B. subtilis* at 30º- 35°C for 7 days.

3.3.2 *Issatchenkia orientalis* 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. This control is used to confirm that a sufficient number of viable organisms were added to the 11th Vessel Positive Controls.

Note: Incubate plates inoculated with *I. orientalis* at 20º- 25°C for 14 days.

3.3.3 Dilution of Preservative Screening (11th Vessel Positive Control): For each serial tested, inoculate an additional four petri dishes with the appropriate volume 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 Media Volume Limits per Vessel: The maximum volume of media per petri dish will not exceed 125 mL. Volumes greater than 125 mL will be divided evenly into two or more petri dishes and the inoculum volume will be divided accordingly.

3.3.5 Negative Controls: Pour 20-25 mL of supplemented BHIA used in the testing session into two petri dishes. This control is used to confirm the sterility of the media batch according to 9 CFR 113.25(c). Inoculate 2 petri dishes with 0.2 mL of the 1:100 dilution of penicillinase (**Section 3.4.4**). Incubate 1 petri dish from each set of the above negative controls with the serial test vessels, two plates per incubation temperature.

3.4 Preparation of the samples

3.4.1 Refer to the product Outline of Production (OP) for each serial to determine the volume of BHIA medium needed for each test serial. Order a

sufficient volume of BHIA medium to accommodate the test serial(s), positive controls, and negative controls.

3.4.2 For products without accompanying diluent, order sterile purified water in serum vials in volumes determined by the dosage of the product, see **Appendix II**.

3.4.3 Preparation of the BHIA medium: On the day of the test, melt the BHIA 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 60°C. The temperature of the bead bath should be approximately 60°C when the agar is ready.

3.4.4 Immediately prior to testing, prepare a working solution of 100,000 Kinetic (Kersey) units of penicillinase/mL by adding 1.0 mL of penicillinase concentrate (**Section 2.2.5**) into 99.0 mL of sterile water. Just prior to using the BHIA medium, add a volume of penicillinase working solution to the medium to yield a concentration of 500 Kinetic (Kersey) units of penicillinase/mL.

3.4.5 At least ten vials of final product are required for sterility testing.

3.4.6 Label test vessels accordingly.

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.

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

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

4.5 Using a syringe and needle or Vacutainer® needle, rehydrate each lyophilized sample container with water or the accompanying diluent at the volume as specified in **Appendix II**. Thaw frozen samples at room temperature or as instructed in the OP.

4.6 Withdraw product from each container with a new sterile syringe and needle. Dispense the appropriate volume of inoculum, as specified in **Appendix II,** from each vial into two petri dishes (one dish per incubation temperature).

Note: The volume of sample inoculum is determined by a dose per dilution ratio so that 10 doses are placed in each petri dish. The volume of inoculum may range from 0.1 to 0.3 mL. Inoculum volumes greater than 0.3 mL may make the agar too soft to setup properly and may, therefore, be divided among additional petri dishes.

4.7 Dispense the volume of supplemented BHIA medium specified in the OP into each of the 20 dishes per 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 the appropriate incubator.

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

4.9 Once all the test serials have been put on test, prepare the negative controls for the testing session (see **Section 3.3.5**). Pour 20 to 25 mL of BHIA on each negative control plate. Swirl gently to mix and allow the agar to cool in the BSC. Once the agar has hardened, invert the petri dishes and place in the appropriate incubator.

Note: The 11th vessel positive control is not conducted with MS.

4.10 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**, **3.3.2**, and **3.3.3**).

4.10.1 Inoculate four additional plates for the 11th vessel positive controls (see **Section 3.3.3**).

4.10.2 The inoculum for the 11th vessel positive control should be taken from the tenth vial of each serial. If the rehydrated liquid or thawed frozen liquid in the vial is 0.5 mL or less, the inoculum is obtained from an eleventh vial of product.

4.10.3 Dispense the volume of supplemented BHIA medium specified in the OP into each of the inoculated 11th vessel dishes. 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 in the appropriate incubator.

4.10.4 Inoculate two additional petri dishes per indicator organism with approximately 100 CFU to serve as positive controls for the test session (see **Sections 3.3.1** and **3.3.2**). Dispense 20-25 mL of supplemented BHIA 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 and place in the appropriate incubator.

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

4.12 Incubate test vessels placed in the 30°- 35°C incubator for 7 days and test vessels placed in the 20°- 25°C incubator for 14 days.

4.13 On day 7, determine the number of CFUs on plates incubated at 30°- 35°C; record the counts on the test worksheet.

4.13.1 For each serial, average the CFUs of the ten plates containing product only.

4.13.2 For each serial, average the CFUs of the two 11th vessel positive controls.

4.13.3 Average the CFUs for the two plates containing the *B. subtilis* positive control.

4.13.4 Make slides and Gram stain all colony types observed and record the observations on the test worksheet.

4.14 On day 14, determine the number of CFUs on plates incubated at 20°- 25°C; record the counts on the test worksheet.

4.14.1 For each serial, average the CFUs of the ten plates containing product only.

4.14.2 For each serial, average the CFUs of the two 11th vessel positive controls.

4.14.3 Average the CFUs for the two plates containing the *I. orientalis* positive control.

4.14.4 Make slides and Gram stain all colony types observed and record the observations on the test worksheet.

4.15 On the final day of the test, determine the CFUs per dose (CFUs/D) for each test serial by dividing the average CFUs at each temperature by 10 doses (each plate inoculum equals 10 doses). Record the results for each incubation temperature as CFUs/D.

5. Interpretation of the Test Results

5.1 Criteria for a valid test:

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

5.1.2 For final products tested for dilution of preservative at the Center for Veterinary Biologics, the BHIA plates containing *B. subtilis* must contain an average count of 93-128 CFU and the BHIA 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.

5.1.3 For final products tested for dilution of preservative (11^{th} vessel positive control), the average colony count for each indicator organism with the product must be within 20% of the average colony count of the corresponding positive control.

5.1.4 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 (refer to **Section 5.6** for further clarification).

5.2 If the average CFUs/D is less than or equal to 1 at both incubation temperatures, the serial is satisfactory (SAT).

5.3 If the average CFU/D is greater than 1 at either incubation temperature, the test may be repeated using 20 unopened vials. If a retest (RT) is not conducted, the serial or subserial is unsatisfactory (UNSAT).

5.3.1 When additional samples are received for the RT, determine if the diluent batch is the same as the one used in the initial test (if applicable). If the diluent serial numbers match, conduct a sterility test on the 20 vials of diluent by 9 CFR 113.26. Conduct the RT on the product using sterile water as the diluent.

5.3.2 If the diluent provided with the RT samples is absent or from a different batch than that provided in the initial test, discard the provided diluent and conduct the RT using sterile water.

5.4 If the average CFUs/D in the RT is less than or equal to 1 at both incubation temperatures, the serial is SAT.

5.5 If the average CFU/D in the RT is greater than 1 at either incubator temperature, the serial is UNSAT.

5.6 If the average CFUs/D for both incubation temperatures is SAT, but the 11^{th} vessel positive control average CFU is less than 20% for either indicator organism compared to the corresponding Positive control, there will be a 3-week delay before the

test results are reported (*Center for Veterinary Biologics Notice No. 09-25,* https://www.aphis.usda.gov/animal_health/vet_biologics/publications/notice_09_25.pdf), so that a dilution of preservative study can be conducted on the serial according to 9 CFR 113.25(d). If the volume of media listed in the OP for the product indicates interference in the 9 CFR 113.25(d) test, this testing will be reported as unsatisfactory (UNSAT) and the sterility test result will be reported as a no test (NT).

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 113.27(e), U.S. Government Printing Office, Washington, DC.

7.2 The United States Pharmacopeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Easton, Pennsylvania.

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 .06
- Updated cover page.
- Adjusted Bacillus subtilis acceptable validity range.

Version .05
- Updated cover page.
- Updated Sections 1 through 5.

Version .04
- The Bacteriology Section Leader has been updated.
- Updated **Sections 3.3**, **4**, and **5.1**.

Version .03
- **Sections 1-5** have been updated to reflect current practices and clarification.
- **7.3:** Reference added for name change of *Candida krusei* to *Issatchenkia orientalis*.

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Version .02

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While only one significant change was made that impacts the outcome of the test, the following changes were made to the document:

- The document number has been changed from STSAM0909 to SAM 909.
- The Contact has been changed from Gerald Christianson to Sophia Campbell.
- **2.1:** The Bunsen burner has been removed from the list of equipment that is needed for the test.
- **2.1.4:** A 60°C incubator has been added as an alternative to the waterbath.
- **2.2:** The disinfectant has been removed from the list of reagents/supplies that is needed for the test.
- **3.3.2:** This section has been added to describe the 11th vessels positive controls. This is considered a significant change and the interpretation is in **Section 5.5**.
- **3.3.7:** This section has been added to explain environmental controls.
- **3.3.8:** This section has been added to clarify media volume limits used in this test.
- **5.5:** This section has been added as a clarification on how to interpret the 11th vessels positive controls results. This is a significant change that was published in CVB Notice 09-02.
- **Appendix I:** Medium storage conditions have been added.
- **Appendix II:** The diluent size/dosage and inoculum size/plate for a frozen sample have been added.

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Appendix I

Brain Heart Infusion Agar (BHIA) – National Centers for Animal Health (NCAH) Media #10204

Brain Heart Infusion Agar	52 g
QH_2O	1000 mL

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

Appendix II

Diluent size/dosage and inoculum size/plate

DILUENT SIZE	DOSAGE	INOCULUM SIZE/PLATE
2 mL	100	.2 mL
5 mL	500	.1 mL
10 mL	500	.2 mL
10 mL	1000	.1 mL
15 mL	500	.3 mL
30 mL	1000	.3 mL
50 mL	5000	.1 mL
60 mL	2000	.3 mL
75 mL	2500	.3 mL
100 mL	10000	.1 mL
200 mL	20000	.2 mL
250 mL	25000	.1 mL
300 mL	10000	.3 mL
80 mL	8000	.1 mL
150 mL	15000	.1 mL
100 mL	10000 (frozen)	.2 mL