

**United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol**

SAM 910

Supplemental Assay Method for Detection of *Mycoplasma* Contamination

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Supplemental Assay Method for Detection of *Mycoplasma* Contamination

1. Introduction

This Supplemental Assay Method (SAM) describes the test procedures used to detect *Mycoplasma* contamination in live viral products, master cell stocks, and master seed viruses, as prescribed in title 9, *Code of Federal Regulations* (9 CFR), section 113.28. If *Mycoplasma* contamination is present, colonies will form on the agar as seen under a stereoscope.

2. Materials

2.1 Equipment/instrumentation

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

- 2.1.1 Laminar-flow Class II biosafety cabinet (BSC)
- 2.1.2 Dissecting stereoscope (35 – 100X magnification)
- 2.1.3 33°- 37°C incubator (humidified 4-6% CO₂) for agar plate incubation
- 2.1.4 33°- 37°C incubator for broth incubation and anaerobic jar
- 2.1.5 Anaerobic jar system (Oxoid AnaeroJar 3.5 L system and components)
- 2.1.6 Vacuum pump (Gast Manufacturing Corporation)
- 2.1.7 Schrader valve chuck fitted to a 24-inch length of rubber hose
- 2.1.8 Compressed gas regulator
- 2.1.9 Vortex mixer

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

- 2.2.1 *Mycoplasma* broth (**Appendix I**), National Centers for Animal Health (NCAH) Media #10162
- 2.2.2 *Mycoplasma* agar (**Appendix II**), NCAH Media #10167
- 2.2.3 DPN/L cysteine (**Appendix III**), NCAH Media #30039
- 2.2.4 *Mycoplasma hyorhinis* (ATCC #17981)

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- 2.2.5 *Acholeplasma laidlawii* (ATCC #23206)
- 2.2.6 Compressed anaerobic gas mixture (5% CO₂ in 95% N₂)
- 2.2.7 70% ethanol
- 2.2.8 1% Virkon disinfectant solution
- 2.2.9 Sterile water
- 2.2.10 Lab coat and gloves
- 2.2.11 Sterile gauze pads, 4 x 4-inch
- 2.2.12 Sterile syringes and needles
- 2.2.13 Sterile serological pipettes, various sizes
- 2.2.14 Pipet-aid

3. Preparation for the Test

3.1 Personnel qualifications/training

The personnel performing the test 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. The personnel must also have knowledge of safe operating procedures, policies, and guidelines; 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 the BSC on 30 minutes prior to the test session.
- 3.2.3 Monitor temperature of incubators, freezers, and coolers according to SOPs.

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3.3 Preparation of reagents/control procedures

Use *M. hyorhinitis* and *A. laidlawii* as the indicator organisms to determine the growth-promoting qualities of the *Mycoplasma* broth and agar as required in the 9 CFR 113.28(d)(4). Use *M. hyorhinitis* as the positive control for the test procedure.

Note: Preferably, handle the control cultures after preparing the test samples and negative controls to prevent contamination of the samples being tested. If this is not possible, thoroughly disinfect the BSC and supplies before commencing to **Section 4**.

3.3.1 Thaw the frozen vials of *M. hyorhinitis* and *A. laidlawii* stock cultures in the BSC. Rehydrate lyophilized stock cultures with *Mycoplasma* broth according to the reagent data sheet. Mix the stock cultures thoroughly by vortexing immediately prior to use.

3.3.2 Prepare working dilutions of the indicator organism stock cultures according to the reagent data sheet specifications. Mix the dilutions thoroughly by vortexing. Prepare a sufficient volume of each working dilution for each indicator organism (i.e., 9-20 mL volumes of each of the working dilutions).

3.3.3 Test the broth for growth-promoting qualities.

3.3.3.1 Inoculate each of 10 tubes containing 9 mL of *Mycoplasma* broth with 1 mL of the dilution that contains 10 CFU/mL of *A. laidlawii*.

3.3.3.2 Inoculate each of 10 tubes containing 9 mL of *Mycoplasma* broth with 1 mL of the dilution that contains 100 CFU/mL of *A. laidlawii*.

3.3.3.3 Incubate the cultures for 10 days at 33°- 37°C.

3.3.3.4 On day 10 of the test, read the test vessels as positive for growth if a dark precipitate is seen on the bottom of the vessel. Record growth as the number of positive per 10 vessels for each dilution.

3.3.4 Test the agar for growth-promoting qualities and verify the concentration of the *M. hyorhinitis* working dilutions.

3.3.4.1 Inoculate four agar plates with 0.1 mL of the dilution of *M. hyorhinitis* that contains 10 CFU/mL.

3.3.4.2 Inoculate four agar plates with 0.1 mL of the dilution of *M. hyorhinitis* that contains 100 CFU/mL.

3.3.4.3 Tilt the plates in a circular motion to allow the inoculum to flow over the surface. Make a short continuous “Z” streak across the

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agar surface with a pipette. Incubate two plates of each dilution under anaerobic conditions (**Appendix IV**) and the other two plates in a humidified 4-6% CO₂ incubator.

3.3.4.4 Examine the agar plates after 10-14 days of incubation.

3.4 Preparation of the samples

3.4.1 Obtain sufficient *Mycoplasma* broth and agar as needed for the test.

3.4.2 Refer to the Outline of Production (OP) for specific testing instructions for each sample.

3.4.3 Number the media test vessels and agar plates to coincide with the samples and controls to be tested.

4. Performance of the Test

4.1 Dress for the *Mycoplasma* test by wearing a lab coat and gloves.

4.2 Wipe down the interior surfaces of the BSC used for testing with 70% ethanol prior to placing supplies, equipment, samples, control cultures, and media in the BSC.

4.3 Aseptically add 2 mL of DPN/L cysteine to each flask of *Mycoplasma* broth that will be used for testing.

4.4 Thaw frozen products immediately before testing. Immediately prior to opening a sample container, disinfect the surface of sample vials and ampules with a 4 x 4-inch gauze pad soaked in 70% ethanol in the BSC. Rehydrate lyophilized products according to the OP with *Mycoplasma* broth. Dilute poultry vaccines that are to be administered by drinking water with 30 mL *Mycoplasma* broth per 1,000 doses, or as indicated in the OP.

4.5 Mix the sample thoroughly and then place 1 mL of the product into a flask containing 100 mL of supplemented *Mycoplasma* broth. Close the flask and mix the culture thoroughly by swirling.

4.6 Place 0.1 mL of product on each of two agar plates. Tilt each plate in a circular motion to allow the inoculum to flow over the surface. Make a short continuous “Z” streak across the agar surface with a pipette. Leave the plates in the closed, upright position in the BSC until the inoculum has been absorbed.

4.7 Repeat the procedures in **Sections 4.4 through 4.6** for the remaining samples to be tested.

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- 4.8** Prepare the control vessels for the test session.
- 4.8.1.** For the negative control, incubate one flask containing 100 mL of uninoculated *Mycoplasma* broth supplemented with DPN/L cysteine. To confirm purity of the supplemented broth at the initiation of the test, place 0.1 mL of uninoculated supplemented broth on each of two agar plates. Tilt each plate in a circular motion to allow the inoculum to flow over the surface. Make a short continuous “Z” streak across the agar surface with a pipette. Leave the plates in the closed, upright position in the BSC until the inoculum has been absorbed.
- 4.8.2.** For the positive control, inoculate one flask containing 100 mL of *Mycoplasma* broth supplemented with DPN/L cysteine with 1 mL of the *M. hyorhinis* dilution that contains 100 CFU/mL.
- 4.9** Incubate the test mediums.
- 4.9.1.** Incubate all culture flasks from the testing session in a 33°- 37°C incubator for 14 days.
- 4.9.2.** Incubate half of the agar plates inverted and covered in a humidified 4-6% CO₂ incubator for 10-14 days.
- 4.9.3.** Incubate the other half of the agar plates inverted in an anaerobic jar, prepared as described in **Appendix IV**, and placed in a 33°- 37°C incubator for 10-14 days.
- 4.10** Subculture the broth cultures on post-inoculation days 3, 7, 10, and 14.
- 4.10.1.** Handle the cultures in the following order to prevent cross-contamination: negative control, test samples, and positive control.
- 4.10.2.** Swirl each flask of broth immediately prior to dispensing to thoroughly mix the culture.
- 4.10.3.** Place 0.1 mL of culture on each of two agar plates. Tilt each plate in a circular motion to allow the inoculum to flow over the surface. Make a short continuous “Z” streak across the agar surface with a pipette. Leave the plates in the closed, upright position in the BSC until the inoculum has been absorbed.
- 4.10.4.** Incubate the mediums as indicated in **Section 4.9**.
- 4.11** At 10-14 days postinoculation, examine the agar plates for *Mycoplasma* colonies using a stereoscope at 35 – 100X magnification.

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4.12 After each test session, disinfect the interior of the BSC and counter tops with 70% ethanol. Discard the biological samples and any extra media according to SOPs.

5. Interpretation of the Test Results

5.1 If *Mycoplasma* colony growth appears on any of the positive control plates and does not appear on any of the negative control plates, the test is valid.

5.2 If no *Mycoplasma* colonies are detected on any of the sample agar plates, the sample is determined to be negative for *Mycoplasma* contamination and is satisfactory (SAT).

5.3 If *Mycoplasma* colony growth is found on any of the sample agar plates, the sample is positive for *Mycoplasma* contamination and is unsatisfactory (UNSAT).

5.4 If mold or bacteria is detected on the agar plates at any time during the test and the sample cannot be ruled out as the source of the contamination, a sterility test will be performed according to the appropriate SAM. This will add a 3-week delay before the test results are reported for the serial. If the sterility test indicates that the serial is contaminated, the Center for Veterinary Biologics (CVB) will report a No Test (NT) for the *Mycoplasma* test and an UNSAT for the sterility test. The final serial disposition will be reported as UNSAT.

6. Report of Test Results

Report the test results as described by SOPs.

7. References

Title 9, *Code of Federal Regulations*, section 113.28, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .05

- The coversheet has been updated.

Version .04

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- The Bacteriology Section Leader, the CVB-PEL Director, and page 1 were updated.
- Updated **Section 2**.
- Deleted approximately from the entire document.
- **Sections 3.3 and 5.4:** Rewritten for clarification.
- **Appendix I:** Updated media storage limit.

Version .03

- Updated and reorganized **Sections 2-5** to reflect current practices and procedures.
- **Appendix I and Appendix III:** Updated storage limits and preparation protocols of media.
- Added **Appendix IV** to describe the use of an anaerobic jar to produce anaerobic conditions for *Mycoplasma* spp. growth.

Version .02

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. One significant change was made that might impact the designation of the serial but not the *Mycoplasma* test; the following changes were made to the document:

- The Contact information has been updated.
- **2.1:** The Bunsen burner has been removed from the list of equipment that is needed for the test. The disinfectant has been removed from the list of equipment that is needed for the test. Clarification on the type of incubators needed has been added.
- **2.2.5/2.2.6/2.2.7:** The tubes, flasks, and petri dishes have been separated out into individual items and information on the sizes used has been added.
- **3.3.2:** The concentration of the inoculum used for testing has been clarified and the growth promotion testing from sample preparation has been separated into another statement.
- **4.8:** Preparation of the inoculum has been rewritten for clarity.
- **4.13:** The references have been clarified.

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- **5.1:** The test conclusions have been separated into individual items and clarified.
- **5.4:** Follow-up testing (conducted in accordance with CVB Notice 09-02) has been added. This change will not impact the outcome of the *Mycoplasma* test but the follow-up sterility test could impact the overall disposition of the serial on test.
- **Appendices:** Media storage parameters have been added.

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Appendices

Appendix I

Mycoplasma (MG) Broth – National Centers for Animal Health (NCAH) Media #10162

Heart Infusion Broth	25 g
Proteose Peptone #3	10 g
Yeast Extract	5 mL
1% Thallium Acetate	25 mL
1% Tetrazolium Chloride	5.5 mL
Penicillin (100,000 U/mL)	5 mL
Horse Serum (Heat inactivate.)	100 mL
QH ₂ O	970 mL

Adjust pH to 7.9 with 10% NaOH.

Filter through 0.2- μ m sterilized mini capsule filter and dispense 100 mL volumes into sterilized glass 125-mL flasks, 9 mL volumes in sterile 16 x 125-mm screw-capped tubes, and 30 mL volumes in sterile 50-mL screw-capped tubes.

Media must be prepared in a sterile room. Media may be stored up to 3 months at 20°- 25°C.

Appendix II

Mycoplasma (MG) Agar – NCAH Media #10167

Heart Infusion Agar	25 g
Heart Infusion Broth	10 g
Proteose Peptone #3	10 g
1% Thallium Acetate	25 mL
QH ₂ O	995 mL

Heat to boiling. Cool and adjust pH to 7.9 with 10% NaOH. Autoclave 20 minutes.

Cool to 56°C and add:

Horse Serum (HI)	126 mL
Yeast Extract (Sterile)	5 mL
0.5% Penicillin	5.2 mL
1% DPN-Cysteine	21 mL
TOTAL (1152 mL)	

Dispense 12 mL in sterile 15 x 60-mm petri dishes. Media must be prepared in a sterile room. Media may be stored up to a month at 20°- 25°C.

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Appendix III

DPN/L-Cysteine – NCAH Media #30039

Nicotinamide-adenine-dinucleotide (DPN, NAD) 5 g
Q.S. H₂O to 500 mL
L-Cysteine 5 g
Q.S. H₂O to 500 mL

Mix each chemical separately until dissolved. Pour solutions together and let mix. Filter and dispense 10 mL volumes in sterile 16 x 150-mm screw-capped tubes. Media may be stored up to a year at -20°C.

Appendix IV

An anaerobic jar is used to maintain anaerobic conditions for a variety of fastidious *Mycoplasma* species. The following procedures are used for the Oxoid AnaeroJar 3.5 L System:

1. Remove the clamp, lid with O-ring seal, and the plate rack from the anaerobic jar. Invert the agar plates and stack them in the plate rack. Be sure to use an even number of plates to keep them from tipping over inside the jar. An uninoculated agar plate may be used as an extra plate.
2. Place a small stack of 4 x 4 gauze pads moistened with water on the bottom of the jar to provide humidity during incubation. Place the plate rack with stacked plates back into the anaerobic jar. Place the lid with O-ring seal onto the anaerobic jar and attach the clamp. Turn the knob on the clamp until it is hand-tight.
3. Attach the rubber hose to the vacuum port of the pump. Attach the end of the hose fitted with the Schrader valve chuck to the Schrader valve labeled “vacuum” on the lid of the anaerobic jar. The gage on the lid measures both vacuum and pressure. Vacuum is measured in inches of mercury (*inHg*) and are indicated by red numbers. Pressure is measured in pounds per square inch (*psi*) and is indicated by black numbers. The gauge should read zero (0).
4. Start the vacuum pump and allow the vacuum in the jar to reach 20 to 25 *inHg*. Detach the Schrader valve from the jar lid and then turn off the vacuum pump.
5. Detach the hose from the vacuum pump port and attach it to the N₂/CO₂ gas regulator port. Turn on the N₂/CO₂ gas regulator valve and allow gas to start flowing, and then attach the Schrader valve chuck to the unmarked Schrader valve on the lid of the anaerobic jar.

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- 6.** Allow the pressure of the anaerobic jar to return to 0. Detach the Schrader valve from the jar lid and then turn off the flow of gas.
- 7.** Repeat **Steps 3 through 6** twice.
- 8.** Incubate the anaerobic jar in a 33°- 37°C incubator for 10 to 14 days.
- 9.** After use, disinfect the jar and its components. Liberally spray the inside of the anaerobic jar lid with fresh 1% Virkon solution. Allow the disinfectant to soak for 10 minutes. Rinse the lid with deionized water and air dry.
- 10.** Fill the anaerobic jar with water and deposit two Virkon tablets in the water. Place the plate rack rubber and O-ring seal in the jar. Allow the disinfectant to soak for 30 minutes. Rinse thoroughly with hot water, rinse with deionized water, and air dry.