United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Polymerase Chain Reaction for the Detection of *Mycoplasma* Contamination

Date: January 21, 2025

Reference Number: CVB-PRO-0006.03

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Table of Contents

1. Introduction

This Testing Protocol (PRO) describes a polymerase chain reaction (PCR) assay for the detection of extraneous *Mycoplasma* in biological samples. This assay can be used in conjunction with the culture test specified in title 9, *Code of Federal Regulations* (9 CFR), part 113.28, as detailed in Supplemental Assay Method (SAM) 910, or as a stand-alone test in situations where the 9 CFR test is not required.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below. Equipment should be cleaned and maintained in a manner conducive for performing sensitive nucleic acid assays.

2.1.1 Laminar Flow Biological Safety Cabinet (BSC) with UV light (NuAire Inc., Class II Type A2) or equivalent

Note: At least three BSCs are required for the procedures outlined in this PRO to separate the processes that may cause nucleic acid cross-contamination. Separate BSCs are required for DNA extraction (Section 4.1), master mix preparation (Section 4.2), and extracted sample addition to the PCR master mix (Section 4.3).

2.1.2 Thermocycler (Applied Biosystems, GeneAmp PCR System 9700 or equivalent)

2.1.3 20-watt UV lamp (UVP, model XX-20S) with 368 nm UV tube (Optional, refer to **Step 4.2.8**)

2.1.4 Electrophoresis system (Invitrogen Corporation, E-gel Electrophoresis System)

2.1.5 Single channel pipettes (Rainin Instrument, LLC, Pipet-Lite L2, L10, L20, L100, L200, L1000, or equivalent)

2.1.6 Microcentrifuge (Eppendorf International, series 5424)

2.1.7 Water bath capable of maintaining 70°± 2°C (Thermo Scientific, Precision series 280 or equivalent)

2.1.8 Vortex mixer

2.1.9 Gel Documentation System and Software (Kodak Gel Logic 2200, Carestream Health Molecular Imaging Software, version 5.0 or current version or equivalent instrument and software)

2.1.10 Refrigerator, capable of maintaining 3°- 7°C.

2.1.11 Freezer, capable of maintaining -70°C or colder.

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. Reagents should be prepared, handled, and stored in a manner that prevents cross-contamination with nucleic acids or endonucleases. Supplies should be certified DNAase/RNAase/pyrogen free and handled in a manner conducive for performing sensitive nucleic acid assays.

2.2.1 QIAamp Fast DNA Stool kit (Qiagen, catalog number 51604)

2.2.2 HotStarTaq Plus Master Mix kit (Qiagen, catalog number 203645)

2.2.3 Magnesium chloride solution ($MgCl_2$), 50 mM (Bioline, catalog number BIO-37026)

2.2.4 8-methoxypsoralen (Sigma-Aldrich, catalog number M3501) diluted to 2.5 mg/mL in DMSO (Sigma-Aldrich, catalog number D8418) (optional, refer to **Step 4.2.4**)

2.2.5 DNase/RNase-free distilled water, PCR-grade, sterile (Invitrogen, catalog number 10977-023 or equivalent)

2.2.6 Tris-EDTA (1X TE) buffer, pH 8.0, sterile (Invitrogen, catalog number AM9849 or equivalent)

2.2.7 1.2 or 2% SYBR Safe agarose gels, prepackaged (Invitrogen, catalog numberA42135)

2.2.8 Ethanol, 200 proof molecular grade (Sigma-Aldrich, catalog number E7023 or equivalent)

2.2.9 1kb DNA ladder (Invitrogen, catalog number 10787-018 or equivalent)

2.2.10 10X DNA loading buffer (Life Technologies, catalog number 10816-015)

2.2.11 0.2-mL thin walled clear PCR reaction tubes, sterile (Axygen, catalog number PCR-02D-C or equivalent)

2.2.12 1.5-mL microtubes, DNAse/RNAse and Pyrogen-free, sterile (Axygen, catalog number MCT-150-C or equivalent)

2.2.13 2-mL screw-capped microtubes (Sarstedt, catalog number 72.694.006)

2.2.14 125-mL plastic media bottle (Wheaton, catalog number 219975IP)

2.2.15 Sterile filtered pipette tips, DNAse/RNAse and Pyrogen-free, sizes ranging from 0.1-µL to 1000-µL

2.2.16 PCR tube cooler tray (Eppendorf, PCR cooler)

2.2.17 PCR tube rack (USA Scientific or equivalent)

2.2.18 Microcentrifuge tube rack (USA Scientific or equivalent)

2.2.19 Ice bucket

2.2.20 Disposable lab coat

2.2.21 Disposable nitrile gloves, powder free

2.2.22 pMyco positive control plasmid diluted to 0.5 ng/µL concentration in 1X Tris-EDTA buffer. This reagent is available from the Center for Veterinary Biologics (CVB). Refer to the current reagent data sheet for more information.

2.2.23 Myco primers diluted to 50 pM/µL each (refer to **Section 7, Reference 1** for primer specificity information)

Note: Reconstitution of the primers is determined by the synthesis data sheet. Calculate the volume of sterile 1X Tris-EDTA (TE) or PCR grade water to reconstitute a primer batch as follows:

$$\frac{\text{Total Batch Yield (nM)} \times 1000\ (\text{pM/nM})}{50\ (\text{pM/µL})} = \text{TE Volume (µL)}$$

2.3 Primer sequences:

MycoA: 5' GGC GAA TGG GTG AGT AAC ACG 3'

MycoB: 5' CGG ATA ACG CTT GCG ACC TAT G 3'

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, knowledge of nucleic acid amplification techniques and workflow processes, knowledge of proper handling and disposal of biological agents, reagents, and chemicals, and knowledge of the proper use of personal protective equipment required to handle UV light sources.

3.2 Preparation of equipment/instrumentation

3.2.1 Operate all equipment/instrumentation according to manufacturers' instructions and monitor compliance with current corresponding standard operating policies/procedures (SOPs).

3.2.2 Ensure that all equipment is maintained according to current policies/procedures.

3.2.3 Program the thermocycler with the *Mycoplasma* PCR program. The annealing temperature begins at 70°C and is reduced by 1°C every two cycles until it reaches 60°C (total of 20 cycles), where it remains for an additional 20 cycles. Refer to **Section 7, Reference 1** for more detailed information regarding the amplification program.

	Initial Denaturation:	5 minutes at 95°C
Cycles 1-20	Denaturation:	30 seconds at 94°C
	Annealing:	30 seconds at 70°C → 60°C
	Elongation:	45 seconds at 72°C
Cycles 21-40	Denaturation:	30 seconds at 94°C
	Annealing:	30 seconds at 60°C
	Elongation:	45 seconds at 72°C
	Final Elongation:	4 minutes at 72°C
	Hold:	4°C

3.3 Preparation of reagents/control procedures

3.3.1 The pMyco positive control plasmid is a purified plasmid containing a truncated *M. hyorhinis* PCR amplicon that has been produced by the CVB and is available upon request. It should be handled only in a designated BSC with task-designated pipettes and other equipment to prevent cross-contamination. Store the pMyco positive control plasmid in a refrigerator for up to 24 months, or in a freezer for longer storage.

3.3.2 Prepare QIAamp Fast DNA Stool kit reagents, Buffer AW1 and Buffer AW2, according to the manufacturer's instructions.

3.3.3 Prepare the DNA ladder to its optimal dilution in 1X DNA loading buffer ; refer to the manufacturer's instructions. Store at -20°- 25°C.

3.3.4 Record sample information, reagent lot numbers, and other pertinent information as per SOPs.

3.4 Preparation of the sample

Samples of product being tested by the *Mycoplasma* PCR assay may come from a variety of sources including directly from the original product vial, broth enrichment subcultures on test by 9 CFR 113.28, and/or mammalian cell-culture enrichment. The broth enrichment and cell-culture enrichment procedures use positive and negative controls to assess the validity of the system; these controls must be tested by this protocol alongside the sample(s) being investigated. Original product vial samples are tested for PCR inhibition, but broth enrichment samples may also be analyzed if necessary.

3.4.1 Original product vial samples

A replicate sample of original product is spiked with pMyco positive control plasmid to test for inhibitory effects in the DNA extraction process and on the PCR assay.

1. Pipet 0.5 mL of product into two separate screw-capped microtubes. Label the tubes with the sample identity and date.

2. In the BSC dedicated to sample inoculation, spike one replicate sample with 0.5 ng pMyco positive control plasmid.

3. Continue to **Section 4** with both the spiked and non-spiked sample. If necessary, both samples may be stored in a refrigerator for up to 3 days, or in a freezer for longer storage.

3.4.2 Broth enrichment subcultures and cell-culture enrichment samples

Samples from broth enrichment and cell-culture enrichment are taken at the subculturing session of the respective protocol. Refer to **SAM 910**, *Supplemental Assay Method for Detection of Mycoplasma Contamination*, or **CVB-SOP-0109**, *Cell-culture Enrichment of Products for the Detection of Viable Mycoplasma Contamination*.

1. Pipet 0.5 mL of sample culture into three separate screw-capped microtubes. Label the tubes with the sample identity and date.

2. Continue to **Section 4** with one sample vial. Testing of the remaining sample vials is conducted as needed. The samples may be stored in a refrigerator for up to 3 days, or in a freezer for longer storage.

4. Performance of the Test

4.1 DNA extraction

DNA extraction is conducted in a designated BSC with task-designated pipettes. To avoid cross-contamination, handle samples that have been spiked with the pMyco positive control plasmid last and change gloves between steps.

4.1.1 Remove test sample(s) from storage and place in a microcentrifuge tube rack.

4.1.2 Follow the protocol titled "Isolation of DNA from Stool for Pathogen Detection" in the QIAamp Fast DNA Stool kit product manual (March 2014), with the following changes:

1. Skip step 1.

2. In step 2, add 1.0 mL InhibitEX Buffer to each 0.5 mL sample.

3. In step 14, elute DNA with 100 µL purified distilled water.

4. Elution centrifugation is conducted at 9,500 x *g*.

4.1.3 Store DNA in a refrigerator for up to 3 days, or in a freezer for longer storage.

4.2 Master mix preparation

Master mix preparation is performed in a clean BSC with task-dedicated pipettes, pipette tips, reagents, and other equipment. Treatment of the master mix with 8-methoxypsoralen and long-wave UV light is an optional step used to ensure purity of the master mix from contaminating DNA, refer to **Section 7, Reference 2** for further details.

4.2.1 Thaw the HotStarTaq Master Mix, 50 mM $MgCl_2$, purified distilled water, and primers. Vortex each reagent briefly to mix and then centrifuge the tubes briefly to remove droplets from the lid. Hold reagents on ice while in use.

4.2.2 Calculate the total number of reactions needed for the samples and controls, then add one to ensure enough master mix is made. Prepare duplicate PCR reactions for each sample, one PCR reaction for the pMyco positive control plasmid, and one PCR reaction for the assay negative control (no template DNA).

4.2.3 Label the appropriate number of PCR tubes to correspond with each sample and place in PCR tube cooler tray.

4.2.4 Add the master mix ingredients to a 1.5 mL microtube as listed below; adjust volumes according to the number of reactions.

Example (volume per reaction):

Purified distilled water	1.5 µL
HotStarTaq PCR mastermix	25.0 µL
$MgCl_2$ (50 mM)	2.0 µL
MycoA (50 pM)	0.5 µL
MycoB (50 pM)	0.5 µL
8-methoxypsoralen (2.5 mg/mL)	0.5 µL

Note: Addition of 8-methoxypsoralen to the master mix is optional. This chemical is used in conjunction with 368 nm UV treatment to eliminate cross-reacting contaminants in the master mix. If not used, add an additional 0.5 µL water per reaction to the master mix.

4.2.5 Pulse vortex the master mix for 10 seconds and centrifuge briefly to remove droplets from the lid.

4.2.6 Dispense 30 µL of master mix into each labeled PCR tube, close the lid.

4.2.7 Add 20 µL purified distilled water to the tubes assigned as assay no-template control and the pMyco positive control.

4.2.8 Lay the tubes horizontally on the cabinet work surface. Expose the dispensed master mix to 368 nm UV light for exactly 4 minutes.

Note: Do not expose unprotected eyes or skin to shortwave UV light as rays can cause severe burns. PCR master mix exposure to 368 nm UV is an optional step used in conjunction with the addition of 8-methoxypsoralen to the master mix. If not used, proceed from Step 4.2.7 to Step 4.2.9.

4.2.9 Place the reaction tubes in a PCR tube cooler tray.

4.3 Sample inoculation

Extracted sample addition to the PCR master mix is performed in a dedicated BSC with task-dedicated pipettes and pipette tips for sample DNA and the pMyco positive control plasmid.

4.3.1 In the extracted sample addition BSC, add 20 µL of sample DNA to each corresponding PCR tube. Close the lid after adding DNA, and change pipette tips between each PCR tube.

4.3.2 Add 0.5 ng pMyco positive control plasmid to the corresponding PCR tube.

4.4 Amplification

The thermocycler is located away from PCR-dedicated BSCs and the analysis area.

4.4.1 Start the *Mycoplasma* PCR program on the thermocycler, set the reaction volume to 50 µL.

4.4.2 Place the PCR reaction tubes in the thermocycler tube tray and close the heated cover.

4.4.3 When the amplification program is complete, remove the PCR reaction tubes and briefly centrifuge in a dedicated microcentrifuge to remove droplets from the lid. The PCR reactions may be stored in a refrigerator up to 3 days until ready to analyze by electrophoresis.

4.5 Electrophoresis of PCR reactions

Electrophoresis of PCR reactions is conducted on a bench top with task-dedicated pipettes and pipette tips. PCR products are stored separately from sample DNA locations.

4.5.1 Follow manufacturer's instructions for preparing the 2% agarose gel and operating the electrophoresis system. Refer to **Section 2.2.7** for safe handling of pre-packaged agarose gels.

4.5.2 Pipette 20 µL of PCR sample into the corresponding well in the agarose gel.

4.5.3 Pipette 20 µL of diluted DNA ladder into the corresponding well of the agarose gel.

4.5.4 Pipette 20 µL purified distilled water into any unassigned well(s) of the agarose gel.

4.5.5 Begin the 30-minute electrophoresis run according to the manufacturer's instructions.

4.6 Visualization and documenting electrophoresis gel

4.6.1 When the gel has finished its electrophoresis run, disassemble the gel from the electrophoresis system.

4.6.2 Place the gel in the UV light box of the Gel Documentation System.

4.6.3 View and photograph the gel using the Gel Documentation System and Software. Refer to the product user manual for analysis and documentation features.

4.6.4 Save an electronic file of the photograph and analysis, and print if needed.

4.6.5 Dispose of the gel as Hazardous Waste according to SOPs.

4.7 Amplicon analysis

4.7.1 Compare any visible amplicons to the DNA ladder using the Gel Documentation System and Software.

4.7.2 Determine the size of the amplicon using the software's analysis feature. Amplicons within 5% (the margin of error may be determined for each laboratory) of the expected molecular weight are considered positive.

1. The expected size range of *Mycoplasma* amplification is 438-470 base pairs (bp). This range is based on the variation of the 16S rDNA gene among *Mycoplasma* species. The margin of error would extend beyond this range.

2. The pMyco positive control plasmid has a product size of 378 bp.
3. Samples spiked with the pMyco positive control plasmid have a product size of 378 bp.

Note: The combination of certain samples with the pMyco positive control plasmid can occasionally produce additional non-specific amplicons with a size above and/or below the target amplicon.

5. Interpretation of the Test Results

5.1 Validity criteria

If validity criteria are not met, the PCR assay is considered invalid and shall be repeated two additional times without bias. Validity criteria **5.1.3** and **5.1.4** do not apply to the PCR Inhibition test.

5.1.1 pMyco positive control plasmid has a product size of 378 bp.

5.1.2 The PCR assay no-template control has no detectable amplicon between 438-470 bp.

5.1.3 The enrichment culture positive control has a product size between 438-470 bp.

5.1.4 The enrichment culture negative control has no detectable amplicon between 438-470 bp.

5.2 Test and sample disposition

5.2.1 PCR inhibition test

1. If the validity criteria are met and the original product spiked with pMyco positive control plasmid has a visible amplicon at 378 bp, the sample is considered satisfactory and is reported as negative for inhibitory effects.

2. If the validity criteria are met and the original product spiked with pMyco positive control plasmid does not have a visible amplicon at 378 bp, the sample is considered unsuitable for PCR analysis and is reported as positive for inhibitory effects. The inhibition test may be repeated two times so that at least two of the three tests are in agreement. If a product fails the inhibition test, the sample's broth enrichment cultures may be analyzed by the PCR inhibition test to determine if the sample's inhibitory effects have been eliminated by dilution.

5.2.2 *Mycoplasma* PCR detection test

1. If the validity criteria are met, the sample is negative for inhibitory effects, and the sample does not produce an amplicon between 438-470 bp, the sample is considered satisfactory and is reported as PCR negative for extraneous *Mycoplasma*.

2. If the validity criteria are met and the sample exhibits an amplicon between 438-470 bp in the original vial sample and/or the enrichment cultures, the sample is considered unsatisfactory and is reported as PCR positive for extraneous *Mycoplasma*. The PCR detection test may be repeated two times so that at least two of the three tests are in agreement. At supervisory discretion, further analysis of the sample may be conducted.

5.3 Further analysis of PCR positive samples

5.3.1 A PCR amplicon from an unsatisfactory product may be analyzed for genus/species identification by nucleic acid sequencing.

5.3.2 Compare the results of the PCR assay to that of the 9 CFR 113.28 culture test (if available). If a sample is culture positive and PCR positive, the sample is contaminated with viable *Mycoplasma* and is unsatisfactory. If the sample is culture negative and PCR positive, the product should be further investigated to determine if the product contains viable *Mycoplasma* that is non-cultivatable on agar or is contaminated with non-viable *Mycoplasma*.

1. The broth culture of the sample in question may be passaged in fresh broth according to **CVB-WI-0255**, *Broth Culture Passaging for the Detection of Viable Mycoplasma Contamination*, for detection of viable *Mycoplasma* contamination.

2. The product may be tested by the Cell Indicator assay according to **CVB-PRO-0007**, *Indicator Cell Culture Method for Detecting Mycoplasma Contamination,* for detection of cell-adapted viable *Mycoplasma* contamination.

3. The product may be enriched by cell-culture according to **CVB-SOP-0109** and tested by PCR for confirmation of cell-adapted viable *Mycoplasma* contamination.

6. Report of Test Results

Report the results of the *Mycoplasma* PCR test according to SOPs.

7. References

7.1 Eldering, JA, C. Felten, CA Veilleux, BJ Potts. Development of a PCR method for mycoplasma testing of Chinese hamster ovary cell-cultures used in the manufacture of recombinant therapeutic proteins. Biologicals 32:183-193. 2004.

7.2 Meier A., Sander P., Bottger EC. Protocol 4. Elimination of contaminating DNA within PCR reagents. In: Burke JF, editor. PCR Essential Techniques. New York: John Wiley & Sons; 1996. Page 19.

7.3 QIAamp Fast DNA Stool Handbook for DNA purification from stool samples. QIAGEN, March 2014.

8. Summary of Revisions

Version CVB-PRO-0006.03

- Updated to DT format and numbers.

Version CVB-PRO-0006.02

- Alphanumeric number has changed from BBPRO1006 to CVB-PRO-0006.

Version .03

- **2:** Added UV lamp to list of equipment; changed catalog numbers of DNA extraction kit, PCR buffer kit, and molecular weight ladder; added $MgCl_2$, 8-Methoxypsoralen reagent; and removed 0.5 mL tubes from list of regents/supplies.

- **3:** Changed the initial denaturation time and temperature of the PCR reaction. Extended the expiration of pMyco reagent. Modified instructions for preparing reagents.

- **4:** Modified instructions of DNA extraction. Added details of the optional 8-Methoxypsoralen and UV treatment. Modified sample inoculation instructions and electrophoresis preparation instructions.

- **5:** Clarified the "no-template" control description. Updated criteria to current method. Added reference to new BB work instruction.

- **7:** Added a reference for the 8-Methoxypsoralen and UV treatment.

Version .02

- **4.1.2:** Changed the DNA elution buffer from Buffer AE to purified distilled water.