Table of Contents

1.0 Purpose

This procedure describes the materials and methods for quantitative PCR (qPCR) amplification and detection of various species of Mycoplasma that may be present in a sample using the MycoSEQ Mycoplasma Real-Time PCR Detection Kit.

2.0 Scope

The use of the MycoSEQ Mycoplasma Real-Time PCR Detection Kit as described in this SOP provides the ability to detect more than 90 species, including *M. genitalium*, *M. pirum,* Acholeplasma and Spiroplasma, species not readily detectable using ***SOP 22194 - Quantitative PCR (qPCR) Method for Detection of Mycoplasma Species***.

3.0 Authority and Responsibility

3.1 The Director, Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.

3.2 PA/QC is responsible for training laboratory personnel and documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC personnel are responsible for the performance of this procedure.

3.4 PA/QC is responsible for reviewing the data and documentation of the results of this procedure.

3.5 BQA is responsible for quality oversight of this operation.

4.0 Equipment, Materials and Reagents

4.1 MycoSEQ Mycoplasma Real-Time PCR Detection Kit (BDP PN 31167)

4.2 MicroAmp Optical 96 Well Reaction Plates (BDP PN 21141)

4.3 MicroAmp Optical Adhesive Film (BDP PN 21142)

4.4 1X TE (BDP PN 30267 or BDP-approved equivalent)

4.5 Distilled Water, DNase Free RNase Free (BDP PN 10189 or BDP-approved equivalent)

4.6 1X PBS (BDP PN 30007 or BDP-approved equivalent)

5.0 Safety Procedures for Mycoplasma Positive and Biohazardous Samples

5.1 Treat all samples suspected or known to contain Mycoplasma per ***SOP 26101 - Labeling, Transport, Submission, Storage, and Handling of Biohazardous Materials Within the BDP***.

6.0 Procedure

6.1 Determine the sample composition to determine whether a nucleic acid extraction is required.

6.1.1 Previously purified DNA in low salt (< 100mM) typically does not require extraction prior to amplification.

6.1.2 Samples containing concentrated protein, intact cells and/or high salt or other PCR inhibitors must be extracted prior to amplification.

6.2 An extraction negative control should be performed in which 200 µl of 1X PBS are extracted in parallel with the test samples, as described in ***SOP 22212 - Purification of DNA Using the DNeasy Blood and Tissue Kit*** or ***SOP 22972 – Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions***.

6.3 An extraction spike control may be performed to control for the potential loss of target mycoplasma DNA during the sample extraction step. The MycoSEQ Discriminatory Positive/Extraction Control (or BDP-approved equivalent) may be used to spike a buffer blank sample or 1X PBS as described below.

6.3.1 Completely thaw the MycoSEQ Mycoplasma Real-Time PCR Detection Kit Box 2 MycoSEQ Discriminatory Positive/Extraction Control, 1,000 copies/µL.

6.3.2 Add a 5 µL aliquot of MycoSEQ Discriminatory Positive/Extraction Control to 195 µL of buffer blank sample or 1X PBS.

6.3.3 Extract the extraction control sample in parallel with the test samples, as described in ***SOP 22212 - Purification of DNA Using the DNeasy Blood and Tissue Kit*** or ***SOP 22972 – Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions***.

6.4 Extract all test samples and controls according to ***SOP 22212 - Purification of DNA Using the DNeasy Blood and Tissue Kit*** or ***SOP 22972 – Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions***.

6.5 qPCR Master Mix Preparation – Perform in the "DNA-free" PCR Set-up Laboratory (ATRF: B2180).

6.5.1 Completely thaw the MycoSEQ Mycoplasma Real-Time PCR Detection Kit reagents.

6.5.2 Record the reagent and material part numbers, lot numbers and expiration dates as well as any relevant additional comments on Form 22208-01.

6.5.3 Vortex the reagents.

6.5.4 Prepare a Premix Solution as follows.

Component for Premix Solution	Volume for One 30-µL Reaction
Power SYBR Green PCR Master Mix, 2X	15.0 µL
Mycoplasma Real-Time PCR Primer Mix, 10X	3.0 µL
Water	2 µL
Total Premix Solution Volume	20.0 µL

6.5.5 Prepare sufficient volume of Premix Solution to account for all test samples (3 replicate wells) and controls (2 or 3 wells) with 10% excess to account for losses due to repeated pipetting.

6.5.6 Record the volumes used on Form 22208-02.

6.5.7 Mix the Premix Solution by pipetting up and down.

6.5.8 Add 20 µL of Premix Solution to each well of the 96-well PCR plate that will be used.

6.5.9 Kit Negative Control (water): Add 10 µL of Negative Control (water) to each of 2 wells.

6.6 Sample addition to PCR plate – Perform in the Positive Control Set-up Laboratory (ATRF: B2150)

6.6.1 To the Premix Solution dispensed into each well, add the following:

6.6.1.1 Test Sample: Add 10 µL of unknown sample to each of 3 wells.

6.6.1.2 Inhibition-control Reaction: Add 10 µL of unknown sample + 2 µL of Discriminatory Positive Control to each of 2 wells.

NOTE: The PCR inhibition spike control samples will contain a final volume greater than 30 µL due to the extra spike volume.

6.6.1.3 Positive Control Reaction: Add 2 µL of Discriminatory Positive Control + 8 µL of Negative Control (water) to each of 2 wells.

6.6.1.4 Extraction Positive Control Reaction: Add 10 µL of extraction positive control sample to each of 3 wells.

6.6.1.5 Extraction Negative Control Reaction: Add 10 µL of extraction negative control sample to each of 2 wells.

6.7 Seal the plate with adhesive film. The plate may be kept at room temperature for up to 30 minutes prior to qPCR amplification on the Applied Biosystems SDS 7900HT (***SOP 22901 - ABI Prism® 7900HT SDS Operation and Maintenance)***, the QuantStudio 6 (***SOP 22973 – Operation and Maintenance of the QuantStudio 6 qPCR System***) or a BDP-approved equivalent instrument.

NOTE: Perform in the Positive Control Set-up Laboratory (ATRF: B2150).

6.8 Place the sealed plate into the qPCR instrument ensuring that the plate is appropriately placed in the carriage by lining up the notched corner of the plate to the notched corner of the plate holder.

NOTE: For the Applied Biosystems SDS 7900HT, place a 96-hole compression pad on top of the plate.

6.9 If using the Applied Biosystems SDS 7900HT, select the MycoSEQ SYBR detector setting (Probe reporter dye = SYBR Green, Quencher = non-fluorescent). If using the QuantStudio 6, select SYBR Green Reagents as the reagent type.

6.10 Program the Applied Biosystems SDS 7900HT per ***SOP 22901 - ABI Prism® 7900HT SDS Operation and Maintenance*** or the QuantStudio 6 per ***SOP 22973 – Operation and Maintenance of the QuantStudio 6 qPCR System***, using the following amplification thermal profile:

Temperature	Time (Min:Sec)	Repetition
95°C	10:00	N/A
95°C	00:15	40
60°C	01:00	
95°C	00:15	Melt Curve
60°C	01:00	
95°C	00:15	

6.11 Upon completion of the qPCR run, unload and discard the plate. **Do not remove the adhesive cover to prevent laboratory contamination with the target amplicon!** Retain and reuse the compression pad (Applied Biosystems SDS 7900HT only).

6.12 Save, analyze and print the run results according to ***SOP 22901 - ABI Prism® 7900HT SDS Operation and Maintenance*** or ***SOP 22973 – Operation and Maintenance of the QuantStudio 6 qPCR System***, using a manual Rn threshold of 0.2.

NOTE: Use of other threshold values may be reported with PA Supervisor approval.

7.0 Validity of Results

7.1 Record the assay control results on Form 22208-03.

7.2 The assay validity criteria are as follows:

Control	Ct	Tm
Extraction Negative (Blank) Control	≥36.00	<82°C*
No Template Control (NTC)	≥36.00	<82°C*
Extraction Spike Control	<36.00	82°C-86°C
PCR Positive Control	<36.00	82°C-86°C
Inhibition Control	ΔCt < 3	82°C-86°C

*__NOTE__: Any detected amplification products with a Tm below 82°C may be ignored. These amplification products represent non-specific amplification or primer-dimers and do not interfere with the final results.

7.3 An "Undetermined" or "No Ct" result is a valid result for an Extraction Negative (Blank) Control and a No Template Control (NTC). An "Undetermined" or "No Ct" result occurs when the amplification plot never crosses the cycle threshold.

7.4 The derivative value (DV) from the melt curve analysis should be greater than background for the Extraction Spike Control, PCR Positive Control and Inhibition Control. Include the melt curve for these controls on Form 22208-04.

7.5 The Inhibition Control ΔCt is defined as the mean Ct value for the Inhibition control sample(s) minus the mean Ct value for the qPCR Positive Control.

7.6 At least one of the extraction positive control qPCR replicates must be lower than Ct 36.

7.7 If the run is valid, proceed to Step 8.1 to determine whether the sample is positive or negative for mycoplasma.

7.8 If the run is invalid, the assay may be repeated with supervisor approval.

8.0 Analysis of Results

8.1 Assay results are reported as either "Positive" or "Negative" for the presence of Mycoplasma genomic DNA on Form 22208-05.

8.2 The criteria for determining whether a sample is Mycoplasma Positive or Negative are indicated in the table below:

Result	Ct	Tm
Positive	<36.00	75°C-86°C
Negative	≥36.00	<75°C

8.3 For a sample to be called positive, the Ct value must be <36 and the Tm for the amplification product must be in the range of 75°C to 86°C.

8.4 For a sample to be called negative, the Ct value must be ≥36 or the Tm must be less than 75°C or both.

NOTE: An Undetermined or No Ct result is also considered negative.

8.5 **The derivative value (DV) from the melt curve analysis should be greater than background for a sample to be called positive. Include the melt curve for each sample on Form 22208-04.**

8.6 If the sample is negative, but the inhibition control exhibits a ΔCt that is ≥3, the qPCR reaction was likely inhibited. Indicate this in the comments section of Form 22208-05 and re-purify and re-test the sample. If the sample is mycoplasma-positive but the inhibition control exhibits a ΔCt that is ≥3, the sample is reported as positive for mycoplasma. Indicate this result in the comments section of 22208-05.

9.0 Documentation

Record all reagent part numbers, lot numbers and expiration dates on Form 22208-01.

Record the 96-well plate layout and master mix preparation volumes on Form 22208-02.
Record control results on Form 22208-03.

Record Melt Curves on Form 22208-04

Record sample analysis results on Form 22208-05

10.0 References and Related Documents

SOP 22194 *Quantitative PCR (qPCR) Method for Detection of Mycoplasma Species*

SOP 26101 *Labeling, Transport, Submission, Storage, and Handling of Biohazardous Materials Within the BDP*

SOP 22212 *Purification of DNA Using the DNeasy Blood and Tissue Kit*

SOP 22972 *Operation and Maintenance of the MagNA Pure 24 System for Nucleic Acid Extractions*

SOP 22901 *ABI Prism® 7900HT SDS Operation and Maintenance*

SOP 22973 *Operation and Maintenance of the QuantStudio 6 qPCR System*