Frederick National Laboratory for
Cancer Research, Frederick, MD

Biopharmaceutical Development Program

Standard Operating Procedure

Title: Quantitative PCR (qPCR) Method for Detection of Mycoplasma Species Using the MycoSEQ Detection Kit

SOP Number: 22208

Revision Number: 02

Supersedes: Revision 01

Effective Date: JUN 07 2020

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

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.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

2.0 Scope

The use of the MycoSEQMycoplasma Real-Time PCR Detection Kit as described in this SOP provides the ability to detect more than 90 species, including *M. gen italium , 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 (BOP PN 31167)

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

4.3 MicroAmp Optical Adhesive Film (BOP PN 21142)

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

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

4.6 1X PBS (BOP PN 30007 or BOP-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.***

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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 BOP-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 E xtract 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.***

Mix Preparation - Perform in the "DNA-free" PCR Set-up Laboratory

6.5.1 Completel y tha w 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 30-1,JL Reaction
Power SYBR Green PCR Master Mix, 2X	15.0 µL
Mycoplasma Real-Time PCR Primer Mix, 1OX	3.0 µL
Water	2 µL
Total Premix Solution Volume	20.0 µL

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6.5.5 Prepare sufficient volumeof Premix Solution to account for all test samples and controls with 10% excessto 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 pipettingup 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).

6.6 -- n to PCR plate - Perform in the Positive Control Set-up Laboratory

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.

6.6.1.2 Inhibition-controlReaction: Add 10 µL of unknown sample+ 2 µL of DiscriminatoryPositive Control.

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).

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 BiosystemsSOS 7900HT (**SOP *22901-ABI Prism® 7900HT* SOS *Operation and Maintenance),*** the QuantStudio 6 (***SOP 22973* - *Operation and Maintenance of the QuantStudio 6 qPCR* System**) or a BOP-approved equivalent instrument.

NOTE: Perform in the Positive Control Set-up Laborato

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 SOS 7900HT, place a 96-hole compression pad on top of the plate.

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

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6.10 Program the Applied Biosystems SOS 7900HT per ***SOP 22901 - AB/ Prism® 7900HT SOS 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 SOS 7900HT only).

6.12 Save, analyze and print the run results according to ***SOP 22901 - AB/ Prism® 7900HT SOS 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.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

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	2:36 .00	<82 °C*
No Template Control (NTC)	2:36 .00	<82 °C*
Extraction Spike Control	<36.00	82°C-86°C
PCR Positive Control	<36.00	82°C-86°C
Inhibition Control	I\Ct < 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 *I\Ct* 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 If the run is valid, proceedto Step 8.1 to determine whether the sample is positive or negative for mycoplasma.

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

This procedureis made available throughfederal funds from the National Cancer Institute, NIH, under contract [redacted]

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	2::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 2::36 o r 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 *I\Ct* that is 2::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 *I\ Ct* that is 2::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 (Attachment 1). Record the 96-well plate layout and master mix preparation volumes on Form 22208-02 (Attachment 2). Record control results on Form 22208-03 (Attachment 3). Record Melt Curves on Form 22208-04 (Attachment 4). Record sample analysis results on Form 22208-05 (Attachment 5).

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 BOP*

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

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*

11.0 Attachments

Attachment 1 Form 22208-01, Quantitative PCR Reagents

Attachment 2 Form 22208-02, 96-Well qPCR Plate Map and Master Mix Preparation

Attachment 3 Form 22208-03, MycoSEQ qPCR Assay Validity Criteria Analysis

Attachment 4 Form 22208-04, Melt Curve Analysis

Attachment 5 Form 22208-05, MycoSEQ qPCR Assay Sample Analysis

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 1

Form 22208-01, Quantitative PCR Reagents

FNLCR, BDP
Form No.: 22208-01
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Quantitative PCR Reagents and Equipment

Reagent	Part Number	Lot #	Expiration
MycoSEQ Mycoplasma Real-Time Detection Kit			
96-Well Optical Plates			
Optical Adhesive Covers			
Water/Buffer (type)			
1X TE			
1X PBS			

Instrument	MEF Number	Calibration/PM Due Date
qPCR Instrument		

Comments:

_____

Form Completed By: _____ **Date:** _____

Reviewed and Approved By: _____ **Date:** _____

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Attachment 2

Form 22208-02, 96-Well qPCR Plate Map and Master Mix Preparation

FNLCR, BDP
Form No.: 22208-02
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96-Well qPCR Plate Map and Master Mix Preparation

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Note: unlabeled wells were not used

Master Mix Preparation		
Reagent	Volume for 1 Reaction (µl)	Volume for ____ (Number) Reactions (µl)
PowerSYBR Green PCR Master Mix, 2X	15.0	
Mycoplasma Real-Time PCR Primer Mix, 2X	3.0	
Water	2.0	
Total Premix Solution Volume	20.0	

Pipette(s) Used:	ME#/Serial#	Calibration Due Date

Performed by: _____ Date: _____

Reviewed by: _____ Date: _____

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Attachment 3 (Page 1 of 2)

Form 22208-03, MycoSEQ qPCR Assay Validity Criteria Analysis

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MycoSEQ qPCR Assay Validity Criteria Analysis	
qPCR Instrument Rn value used	
Comments *Include supervisor approval if Rn value other than 0.2 is used.*	☐ NA

Negative Controls		
No Template Controls	**Results**	**Validity Requirements**
Number of NTC Replicates		NA
Number of NTC Replicates that Generate a Ct Value		NA
Mean Tm of NTC Replicates that Generate a Ct Value		<82°C
Mean Ct of NTC Replicates that Generate a Ct Value		≥36.00
NTC Result (Pass/Fail):[1]		☐ Pass ☐ Fail
Extraction Negative Control	**Results**	**Validity Requirements**
Number of Extraction Negative Replicates		NA
Number of Extraction Negative Replicates that Generate a Ct Value		NA
Mean Tm of Extraction Negative Replicates that Generate a Ct Value		<82°C
Mean Ct of Extraction Negative Replicates that Generate a Ct Value		≥36.00
Extraction Negative Control Result (Pass/Fail):[2]		☐ Pass ☐ Fail

[1] *If none of the NTC replicates generates a Ct value, the NTC result is pass.*

[2] *If none of the Extraction Negative Control replicates generates a Ct value, the Extraction Negative Control result is pass.*

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Attachment 3 (Continued Page 2 of 2)

Form 22208-03, MycoSEQ qPCR Assay Validity Criteria Analysis

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MycoSEQ qPCR Assay Validity Criteria Analysis		
Positive Controls		
qPCR Positive Control Results	**Results**	**Validity Requirements**
Number of qPCR Positive Control Replicates		NA
Mean Tm of qPCR Positive Control Replicates		82°C-86°C
Mean Ct Value of qPCR Positive Control Replicates		<36.00
qPCR Positive Control Result (Pass/Fail)		☐ Pass ☐ Fail
PCR Inhibition Spike Results (Post-Extraction Spike)	**Results**	**Validity Requirements**
Mean Inhibitor Control Tm		82°C-86°C
Mean Ct Value of Inhibition Control		NA
Mean Ct Value of qPCR Positive Control		NA
ΔCt (Inhibitor Control Mean Ct - qPCR Control Mean Ct)		ΔCt < 3
PCR Inhibition Result (Pass/Fail)		☐ Pass ☐ Fail
Extraction Spike Control	**Results**	**Validity Requirements**
Mean Spike Control Tm		82°C-86°C
Mean Ct Value of Spike Control		<36.00
Extraction Spike Result (Pass/Fail):		☐ Pass ☐ Fail
MycoSEQ qPCR Assay Validity (Pass/Fail)		☐ Pass ☐ Fail
Comments		
QC Analyst Signature / Date		

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted]

Attachment 4

Form 22208-04, Melt Curve Analysis

FNLCR, BDP
Form No.: 22208-04
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Melt Curve Analysis

Sample Name: _____

Paste Melt Curve Below:

Derivative Value above Background (Yes/No): _____

Comments:

_____

Form Completed By: _____ **Date:** _____

Reviewed and Approved By: _____ **Date:** _____

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Attachment 5

Form 22208-05, MycoSEQ qPCR Assay Sample Analysis

FNLCR, BDP
Form No.: 22208-05
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MycoSEQ qPCR Assay Sample Analysis

Sample Analysis

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

Sample Name/Sample Number	QC Test Request #	Ct Value	Tm	Mycoplasma (Positive/Negative)

Comments:

QC Analyst Signature / Date:

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