MycoSEQ Mycoplasma Detection assay: qPCR system compatibility and AccuSEQ software analysis

Introduction

The Applied Biosystems™ MycoSEQ™ Mycoplasma Detection Kit is a real-time PCR assay designed to detect more than 90 mycoplasma species with high specificity and no cross-reactivity with closely related bacterial species. The assay detects 10 CFU/mL or the genomic equivalent of 10 GC/mL in accordance with regulatory guidance. After validation and regulatory review, customers leveraging a variety of therapeutic product manufacturing platforms have received approval to use the MycoSEQ assay for their mycoplasma lot-release testing.

- **Simplified workflow**—The comprehensive system includes an optimized and fully automatable sample preparation kit, a real-time PCR assay, and instrumentation with dedicated software. A workflow of less than 5 hours provides actionable results, which makes the assay ideal for both lot-release and in-process testing as part of a microbial risk mitigation strategy.
- **Sensitive and specific detection**—The assay detects the genomes of more than 90 species of *Mycoplasma*, *Acholeplasma*, and *Spiroplasma* with as little as 1 genome copy per PCR reaction. The MycoSEQ assay does not detect the closely related bacterial species of *Lactobacillus*, *Streptococcus*, and *Clostridioides*.
- **Instrument compatibility**—The MycoSEQ Mycoplasma Detection Assay can be performed on both the Applied Biosystems™ 7500 Fast Real-Time PCR System and the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System.

This application note is a summary of testing to evaluate the performance of the MycoSEQ assay on a 7500 Fast Real-Time PCR Instrument and a QuantStudio 5 Real-Time PCR Instrument. In addition, the MycoSEQ Mycoplasma Detection Kit was tested to demonstrate the functionality of Applied Biosystems™ AccuSEQ™ Real-Time PCR Detection Software v3.1. The results shown are what the user can typically expect when the product is used, as outlined in the product user guide.

The MycoSEQ assay is an Invitrogen™ SYBR™ Green–based detection assay to determine the presence or absence of mycoplasma DNA. The method has three objective acceptance criteria: cycle threshold (C_t), melting temperature (T_m), and melting temperature peak height derivative value (DV). AccuSEQ Real-Time PCR Detection Software v3.1 was developed to support the commercial MycoSEQ Mycoplasma Detection assay on the QuantStudio 5 Real-Time PCR System.

ThermoFisher SCIENTIFIC

AccuSEQ software v3.1 provides PCR templates that are preset and assay-specific PCR plate layouts. It provides an easy process for users to set up PCR runs, analyze data, and report results. AccuSEQ software v3.1 includes a MycoSEQ assay template for the mycoplasma assay.

Materials and methods

In both studies, test sample DNA was extracted using the Applied Biosystems™ PrepSEQ™ *Express* Nucleic Acid Extraction Kit and the Applied Biosystems™ AutoMate *Express*™ Nucleic Acid Extraction System. Where possible, field positive samples were used for the study; otherwise, sample matrices were spiked with a control template.

Comparability studies were conducted using genomic DNA from *Mycoplasma arginini* and *Acholeplasma laidlawii* spiked into a high-density (>1 x 10^7 cells/mL) CHO cell culture to final concentrations of 100, 50, 20, and 10 genome copies/mL. PCR reactions were prepared, run, and analyzed in triplicate on the 7500 Fast instrument with Applied Biosystems™ AccuSEQ™ Real-Time PCR Detection Software v2.1.2 and the QuantStudio 5 instrument with AccuSEQ Real-Time PCR Detection Software v3.1.

The AccuSEQ software functionality test used purified mycoplasma genomic DNA obtained from ATCC *Acholeplasma laidlawii* (P/N qCRM-23206D) and *Mycoplasma arginini* (P/N qCRM-23838D). Serial dilutions of DNA were made with a solution containing 10 mM Tris-HCl and 0.1 mM ETDA adjusted to pH 8.0 to appropriate concentrations. 10 µL of the diluted DNA was used for each PCR reaction. For testing PCR inhibition, the MycoSEQ Discriminatory Positive Control was included with the mycoplasma DNA in a separate PCR reaction. PCR reactions were run on the QuantStudio 5 instrument with standard settings for the MycoSEQ template provided with AccuSEQ v3.1 software. Replicate reaction plates were prepared in order to supply one plate for each instrument tested.

Multiple test parameters were evaluated to analyze assay performance, including:

- Positive and negative call concordance
- Mean C_t at low, medium, and high concentrations
- PCR efficiency

Results

The MycoSEQ Mycoplasma Detection Kit demonstrated optimal performance for all parameters tested, even for samples with low copy numbers. The assay accurately detected *Mycoplasma* species in both spiked controls and test samples. The performance of the MycoSEQ Mycoplasma Detection Assay was similar on both the QuantStudio 5 and the 7500 Fast qPCR systems.

Instrument comparison using MycoSEQ

Tables 1 and 2 summarize the results for each species tested. Average and standard deviations for C_t, T_m, and DV are shown for triplicate reactions performed on each instrument. The 7500 Fast and QuantStudio 5 instruments utilize different algorithms to calculate DV; therefore, a comparison of these values between the instruments is inappropriate. Note: A DV of 0.8 on the 7500 Fast instrument with AccuSEQ software v2.1 is equivalent to a DV of 0.4 on the QuantStudio 5 instrument with AccuSEQ software v3.1. These results indicate both instruments perform similarly with the MycoSEQ Mycoplasma Detection assay.

MycoSEQ template requirements test of AccuSEQ software

Table 3 lists the test requirements, obtained values, and

Table 1. Comparison of genomic DNA from *M. arginini* spiked into high-density CHO cell culture and run on the 7500 Fast and QuantStudio 5 Real-Time PCR systems.

M. arginini							
Copies/mL	Measurement	7500 Fast instrument			QuantStudio 5 instrument		
		C_t	T_m	DV	C_t	T_m	DV
100	Average	28.25	80.24	2.79	28.61	80.31	1.08
	Standard Dev	0.05	0.20	0.10	0.12	0.11	0.05
50	Average	29.52	80.24	2.70	29.64	80.38	1.00
	Standard Dev	0.17	0.07	0.07	0.35	0.03	0.02
20	Average	30.21	80.24	2.68	30.26	80.31	0.90
	Standard Dev	0.23	0.20	0.07	0.11	0.11	0.03
10	Average	31.85	80.24	2.39	31.85	80.41	0.89
	Standard Dev	0.12	0.07	0.03	0.11	0.01	0.04

Table 2. Comparison of genomic DNA from *A. laidlawii* spiked into high-density CHO cell culture and run on the 7500 Fast and QuantStudio 5 Real-Time PCR systems.

A. laidlawii							
Copies/mL	Measurement	7500 Fast instrument			QuantStudio 5 instrument		
		C_t	T_m	DV	C_t	T_m	DV
100	Average	30.87	80.21	2.47	31.19	80.52	1.31
	Standard Dev	0.06	0.23	0.19	0.09	0.06	0.08
50	Average	32.87	80.39	2.27	33.10	80.35	1.02
	Standard Dev	0.11	0.23	0.06	0.21	0.16	0.05
20	Average	32.66	80.50	2.37	33.02	80.54	1.08
	Standard Dev	0.17	0.14	0.08	0.18	0.08	0.04
10	Average	33.94	80.44	2.15	34.01	80.50	0.96
	Standard Dev	0.08	0.09	0.02	0.21	0.06	0.04

results of testing the MycoSEQ template with AccuSEQ software. The MycoSEQ Mycoplasma Detection assay was used to test the MycoSEQ template.

Assay detection effectiveness was determined with 24 replicates for which 10 copies of the *A. laidlawii* and *M. arginini* genomes were spiked into the PCR reactions. The detection rates for both species were greater than 95% (Table 4).

AccuSEQ software was tested for the ability to detect PCR inhibition. Wells 3 and 4 were identified as demonstrating PCR inhibition when compared to the positive PCR control (data not shown). No PCR inhibition was observed in wells 1 and 2 (Table 5).

The results indicate that the MycoSEQ template is suitable for the assay and data analysis. The presence and absence calls were made automatically. *M. arginini* and *A. laidlawii* were both detected at >95% at 10 genome copies per reaction, and detection of PCR inhibition was demonstrated.

Table 3. MycoSEQ template requirements tested.

Requirement	Obtained value	Pass/fail
AccuSEQ-QS5 solution shall be able to automatically determine the presence or absence of mycoplasma.	Presence or absence calls are automatic	Pass
AccuSEQ-QS5 solution shall be able to detect mycoplasma DNA at 10 genome copies per reaction with a 95% or greater detection rate.	95.8%	Pass
AccuSEQ-QS5 solution shall be able to determine PCR inhibition. Inhibition is indicated by Δ Ct > 2.0000. No inhibition is indicated by Δ Ct < 2.0000.	• ΔC_t = 0.27 without inhibition • ΔC_t = 7.08 with inhibition	Pass

Table 4. Detection of *A. laidlawii* and *M. arginini*.

Species	Number of positive reactions	Detection rate (%)
A. laidlawii	23 out of 24	95.8
M. arginini	23 out of 24	95.8

Conclusions

The performance of the MycoSEQ assay was equivalent on the 7500 Fast and QuantStudio 5 instruments. This study demonstrates that PCR and analysis using the MycoSEQ assay with AccuSEQ software is highly sensitive. The mycoplasma detection rates were similar using either the 7500 Fast or the QuantStudio 5 Real-Time PCR systems. These results indicate that customers can confidently utilize the MycoSEQ Mycoplasma Detection assay with the appropriate version of AccuSEQ software on both the 7500 Fast and QuantStudio 5 instruments to deliver accurate, reliable, and rapid results for routine laboratory testing or microbial risk mitigation strategies.

Table 5. Test results for the MycoSEQ template.

Well	Sample name	Task	C_t	T_m	DV	Call	Call assessment	ΔC_t
1	*M. arginini*	Unknown	32.63	80.93	1.4	Present	Positive	
2	*M. arginini*	IC	24.89	84.99	1.3	Pass	IC pass	0.27
3	Test sample	Unknown	37.65	79.89	0.2	Review	Review DV	
4	Test sample	IC	32.25	85.89	0.6	Fail	Inhibition detected	7.09

applied
biosystems

Ordering information

Product	Cat. No.
MycoSEQ Mycoplasma Detection Kit	4460623
Pharmaceutical Analytics QuantStudio 5 Real-Time PCR System, 96-well, 0.1 mL, laptop	A31671
Pharmaceutical Analytics QuantStudio 5 Real-Time PCR System, 96-well, 0.1 mL, desktop	A31672
AccuSEQ Real-Time PCR Detection Software v3.1	A48509
Applied Biosystems PrepSEQ Express Nucleic Acid Extraction Kit	4466351
AutoMate *Express* Nucleic Acid Extraction System	4467754
AccuSEQ Real-Time PCR Detection Software v2.1.2	A45662
7500 Fast Real-Time PCR System, laptop	4365464
7500 Fast Real-Time PCR System, desktop	4351107

Find out more at **thermofisher.com/mycoseq**

ThermoFisher
SCIENTIFIC