Mycoplasma detection

Sensitive and specific detection of mycoplasma DNA with the MycoSEQ Plus Mycoplasma Detection Kit

Abstract

Rapid, sensitive, and specific detection of mycoplasma contamination is crucial for ensuring the quality and safety of cell therapy products. This application note details a protocol that can help enable preparation and detection of mycoplasma DNA at a sensitivity aligned with the guidance detailed in the European Pharmacopoeia chapter 2.6.7, "Mycoplasmas" [1], starting with test sample volumes as low as 1 mL using the Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection Kit.

The MycoSEQ Plus kit leverages Applied Biosystems™ TaqMan™ Assay chemistry and utilizes a multiplexed pool of oligonucleotides (primers and probes) for amplification and detection of the target *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Mesoplasma*, *Haemoplasma*, and *Ureaplasma* species. The oligonucleotide pool in the assay enables detection of at least 200 inclusion panel species, while excluding detection of other bacterial and host cell species. When combined with the magnetic bead–based Applied Biosystems™ PrepSEQ™ Nucleic Acid Sample Preparation Kit, this assay enables sensitive and specific detection of mycoplasma DNA recovered from samples typical of cell culture manufacturing, including cell-based therapies.

This application note provides detailed insights into the integration of the MycoSEQ Plus kit within a cell therapy production workflow. It highlights the kit's capability with low-volume test samples.

Introduction

Mycoplasmas, a type of bacteria, are potential contaminants of cell cultures that can be difficult to detect with traditional microbiology methods. Regulatory agencies worldwide require that certain biological products, including cell-based therapies, be tested for mycoplasma contamination to ensure quality and safety. US regulatory agencies provide various guidance documents to assist manufacturers in complying with mycoplasma testing requirements. These documents offer detailed recommendations on testing procedures, validation, and regulatory expectations. Here is guidance specifically for cell-based therapies: "We recommend that you perform mycoplasma testing on the product at the manufacturing stage when the test is most likely to detect contamination, such as after pooling of cultures for harvest but prior to cell washing. Testing should be conducted on both cells and supernatant. We recommend that you inform [us] whether there is in-process testing for mycoplasmas during extended culture procedures. Due to the limited dating period of many cellular products, it is frequently not feasible for a sponsor to perform the recommended culture-based assay for release testing. In those cases, we recommend the use of polymerase chain reaction (PCR)-based mycoplasma assays or another rapid detection assay during product development. As part of your BLA, you should submit appropriate data to demonstrate that the PCR or alternative test has adequate sensitivity and specificity" [2].

High-sensitivity detection of mycoplasmas leveraging the MycoSEQ Plus mycoplasma detection assay is achieved using a combination of concentrating mycoplasmas from the test sample, extracting the DNA by an efficient sample preparation method, and detecting the purified DNA with a sensitive and specific qPCR assay.

This application note details a protocol for the MycoSEQ Plus mycoplasma detection system for test samples where input volume is limited. This protocol follows the standard release test protocol, followed by concentration of the purified DNA by ethanol precipitation. With this protocol, the equivalent of 1 mL of test sample is analyzed in the qPCR reaction. This protocol has been tested, leveraging the sensitivity guidance of 10 colony forming units (CFU) or genome copy equivalents per milliliter of test sample detailed in the European Pharmacopoeia chapter on mycoplasmas [1].

Materials and methods

Testing of media samples containing T cells

Mycoplasma genomic DNA was spiked into each sample (10 genome copies per mL (GC/mL), depending on the sample extraction method and sample volume). Samples were processed using both the manual workflow of the Applied Biosystems™ PrepSEQ™ 1-2-3 Mycoplasma Nucleic Acid Extraction Kit and the automated workflow of the Applied Biosystems™ PrepSEQ™ Express Nucleic Acid Extraction Kit, followed by ethanol precipitation to concentrate the final eluate.

Sample preparation

Experiments were designed and executed to demonstrate the MycoSEQ Plus assay's performance at 10 GC/mL in a test sample of 1–3 mL volume. Samples comprised complex matrices containing T cells: fresh Gibco™ CTS™ OpTmizer™ T Cell Expansion Serum Free Medium (SFM), spent T cell medium, or cryopreservation medium (Table 1). Purified gDNA from key *Mycoplasma* species listed in the European, US, and Japanese pharmacopoeias *(M. arginini*, *M. salivarium*, *M. orale*, *M. fermentans,* and *M. pneumoniae*) and the Applied Biosystems™ MycoSEQ™ Plus Discriminatory Positive Control were spiked into samples prior to processing. Samples were processed with either the PrepSEQ kit manual workflow (see user guide for the PrepSEQ Sample Preparation Kits for *Mycoplasma*, MMV, and Vesivirus, Pub. No. 4465957, Rev C) or with automated workflows for the PrepSEQ Express kit (see user guide for the PrepSEQ Express Nucleic Acid Extraction Kit, Pub. No. MAN0016799, Rev A.0) followed by ethanol precipitation to concentrate the final eluate (see section on ethanol precipitation).

Sample preprocessing

Low-volume (i.e., 1–3 mL) samples of the indicated media containing 10^6 T cells were preprocessed by centrifugation at 1,000 x *g* to pellet the T cells. The supernatants, which contained mycoplasmas, were then transferred to new tubes and centrifuged at 16,000 x *g* to pellet the mycoplasmas. The supernatants were discarded, and the mycoplasma pellets were retained on ice. The T cell pellets were resuspended in 300 µL of cell fractionation buffer and centrifuged at 1,500 x *g* to pellet the cellular membranes and nuclei. The cell fractionation buffer supernatants were transferred to the mycoplasma pellets to resuspend them, followed by DNA template spike-in at 10 GC/mL (Table 2).

Table 1. Sample matrices tested.

Sample matrix tested	Description of sample matrix
Fresh CTS medium	Fresh CTS OpTmizer T Cell Expansion SFM (Thermo Fisher Scientific, Cat. No. A1048501)
Spent T cell medium	Spent T cell medium (CTS OpTmizer Pro SFM + OpTmizer Pro supplement + 2.5% CTS Immune Cell SR + GlutaMAX Supplement + L-glutamine)
Cryopreservation medium	CryoStor Cell Cryopreservation Media (50%) CS10 (MilliporeSigma, Cat. No. C2874) + 10% Human Serum Albumin (HSA), Excipient (Nova Biologics, Cat. No. 68982-0643-02) + 40% Plasma-Lyte A Injection pH 7.4 (Andwin Scientific, Cat. No. 2B2544)

Table 2. DNA spike-in concentrations and volumes for sample extraction workflows.

Sample preparation workflow*	Starting sample volume (mL)	DNA spike-in for 10 GC/mL in sample (GC)	EtOH precipitation final elution volume (µL)	DNA in PCR reaction (GC/rxn)	No. rxn per extraction
AE	3	30	36	~7	3
AE	2	20	24	~7	2
AE	1.5	15	12	10	1
ME	3	30	36	10	3
ME	2	20	24	10	2
ME	1	10	12	10	1

* AE: automated extraction, ME: manual extraction.

Automated extraction

The Applied Biosystems™ AutoMate *Express*™ Nucleic Acid Extraction System enables automated recovery of mycoplasma DNA from complex samples. To each of the sample tubes provided in the PrepSEQ Express kit, 300 µL of spent T cell medium (or fresh CTS medium or cryopreservation medium) with or without mycoplasma DNA was added. Samples were loaded into the AutoMate *Express* system for DNA extraction using the PrepSEQ Express kit protocol with 30 min of proteinase K lysis, and eluted into 100 µL of elution buffer.

Manual extraction

Mycoplasma DNA was also extracted manually using the PrepSEQ 1-2-3 Mycoplasma Nucleic Acid Extraction Kit. To each of the 2 mL microcentrifuge tubes, 300 µL of spent T cell medium (or fresh CTS medium or cryopreservation medium) with or without mycoplasma DNA was added. The manual sample extraction method according to the user guide for the MycoSEQ Plus Mycoplasma Detection Kit was followed for the extraction sample preparation, binding, washing, and elution steps.

Ethanol precipitation

Sample eluates from the PrepSEQ kit extractions from the AutoMate *Express* and MycoSEQ Plus systems underwent ethanol precipitation steps to concentrate them. Optimized volume ratios of isopropanol were added to the sample eluates with 0.3 M sodium acetate salt followed by centrifugation at 16,000 x *g* for 15 min at 4°C. The supernatant was removed, and the DNA pellet was washed with 70% ethanol followed by centrifugation at 16,000 x *g* for 5 min at 4°C. The supernatant was then removed, allowing the DNA pellet to air-dry for 5 min. The DNA was then eluted in an appropriate volume (see Table 2) of PrepSEQ elution buffer by incubating at 55°C for 30 min with gentle shaking (~750 rpm).

MycoSEQ Plus mycoplasma detection assay

All samples were tested with the MycoSEQ Plus Mycoplasma Detection Kit using the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, and data analysis was performed using the Applied Biosystems™ AccuSEQ™ Real-Time PCR Detection Software v3.2.1.

qPCR setup and run

Samples for each of the reaction mixtures were prepared as shown in Table 3. The MycoSEQ Plus Mycoplasma Detection Kit contains 2X qPCR Master Mix Plus, 10X qPCR assay mix, a DNA control (discriminatory positive control, DPC), and negative control water (no-template control, NTC). The 96-well, 0.1 mL plate was loaded into a QuantStudio 5 Real-Time PCR System (with 96-well, 0.1 mL block) running AccuSEQ Real-Time PCR Detection Software. The following thermal cycling run parameters were used for qPCR (see Figure 1): 95°C hold for 10 min, then 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. The channels for Applied Biosystems™ FAM™, VIC™, and NED™ dyes were used to detect the mycoplasma target, the DPC, and the internal positive control (IPC), respectively.

Table 3. qPCR setup.

Kit reagents	Volume for one reaction (µL)	Example: volume for 10 reactions (µL) (with 10% overage)
2X qPCR Master Mix Plus	15	165
MycoSEQ Plus 10X Assay Mix	3	33
Negative control	N/A	N/A
Total	**18**	**198**

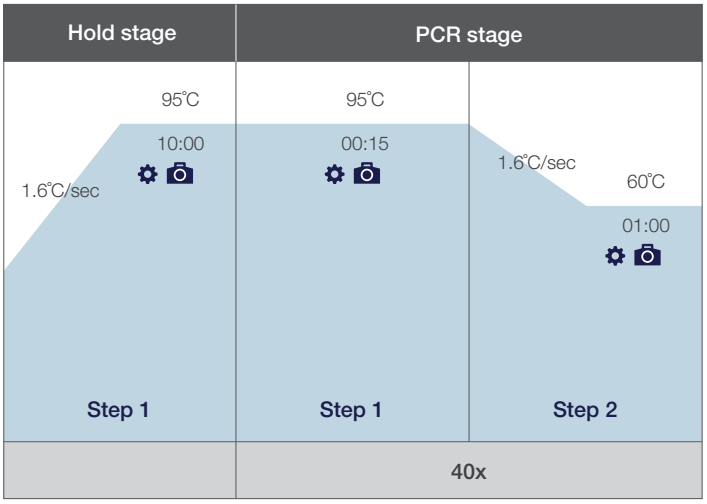

Figure 1. Default qPCR cycling conditions in AccuSEQ software.

Results

The samples were processed by either PrepSEQ manual or automated PrepSEQ Express workflows, followed by ethanol precipitation. PCR was performed on the QuantStudio 5 Real-Time PCR System. The extraction and detection results were analyzed using AccuSEQ Real-Time PCR Detection Software to demonstrate the high sensitivity for key species listed in the European Pharmacopoeia in the complex T cell–containing matrices (Tables 4 and 5, Figures 2–7).

Tables 4 and 5 display details of the samples that were spiked into spent T cell medium or cryopreservation medium, respectively. Five species and the MycoSEQ Plus DPC at 10 GC/mL were spiked into spent T cell medium with 10^6 T cells, and 2 samples and the MycoSEQ Plus DPC at 10 GC/mL were spiked into cryopreservation medium. DNA from samples and DPCs was extracted and then tested and analyzed using the QuantStudio 5 Real-Time PCR System. All species were detected at a 100% rate.

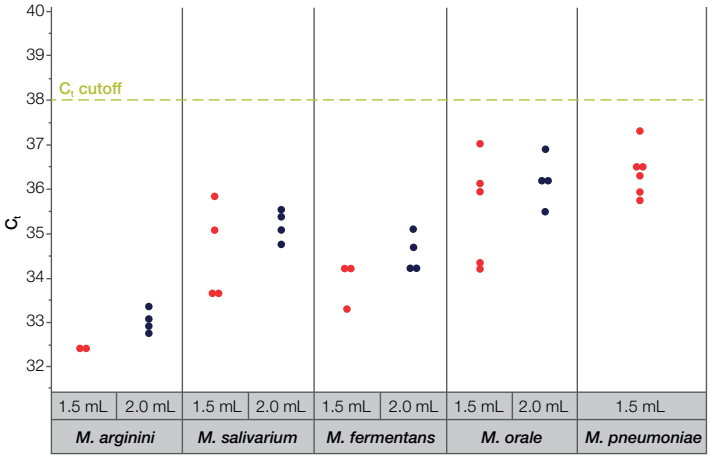

Figure 2. C_t values of *Mycoplasma* species spiked into spent medium and assayed using automated extraction. The scatterplot shows the threshold cycle (C_t) values for gDNA of the noted species spiked into spent medium containing 10^6 T cells at 10 GC/mL and processed using the automated PrepSEQ Express workflow followed by ethanol precipitation. The dotted line represents the C_t cutoff of the assay.

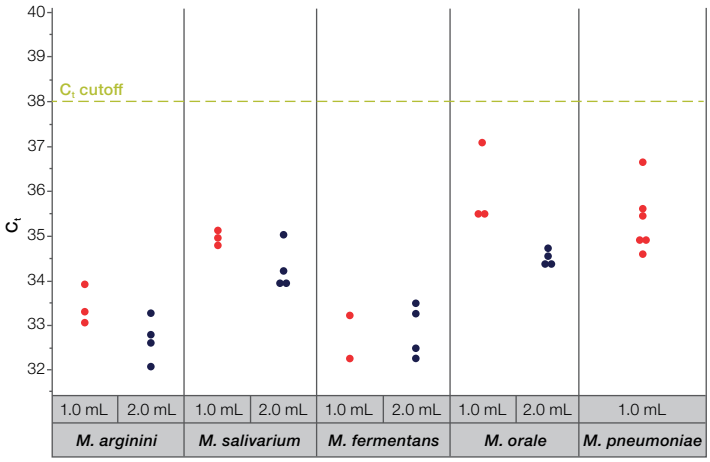

Figure 3. C_t values of *Mycoplasma* species spiked into spent medium and assayed using manual extraction. The scatterplot shows the C_t values for gDNA of the noted species spiked into spent medium containing 10^6 T cells at 10 GC/mL and processed using the PrepSEQ manual workflow followed by ethanol precipitation. The dotted line represents the C_t cutoff of the assay.

Table 4. Comprehensive results of the MycoSEQ Plus detection kit with either the manual PrepSEQ or automated PrepSEQ Express workflow with samples spiked into spent T cell medium.

Species/ target	Copies per PCR reaction	Sample prep method*	Sample volume (mL)	FAM dye mean C_t	S.D.** of FAM dye C_t	CV† (%) of FAM dye C_t	No. replicates	Detection rate (%)
M. arginini	10	AE	1.5	32.4	0.0	0.0	2	100
		ME	1	33.4	0.4	1.3	3	100
	7	AE	2	33.0	0.3	0.8	4	100
	10	ME		32.6	0.5	1.4	4	100
M. salivarium	10	AE	1.5	35.5	0.5	1.5	2	100
		ME	1	35.0	0.1	0.4	3	100
	7	AE	2	35.2	0.3	0.9	4	100
	10	ME		34.3	0.5	1.5	4	100
M. fermentans	10	AE	1.5	33.9	0.5	1.5	3	100
		ME	1	32.7	0.7	2.2	2	100
	7	AE	2	34.6	0.4	1.2	4	100
	10	ME		32.8	0.6	1.7	4	100
M. orale	10	AE	1.5	36.4	0.6	1.6	3	100
		ME	1	36	0.9	2.6	3	100
	7	AE	2	36.2	0.6	1.6	4	100
	10	ME		34.4	0.1	0.3	4	100
M. pneumoniae	10	AE	1.5	36.4	0.5	1.5	6	100
		ME	1.5	35.3	0.7	2.0	6	100
DPC/PEC‡	10	AE	1.5	34.4	0.6	1.7	2	100
		ME	1	35.1	0.7	2.0	3	100
	7	AE	2	33.3	0.5	1.5	4	100
	10	ME		34.9	0.5	1.3	4	100

* AE: automated extraction, ME: manual extraction.
** S.D.: standard deviation.
† CV: coefficient of variation.
‡ PEC: positive extraction control.

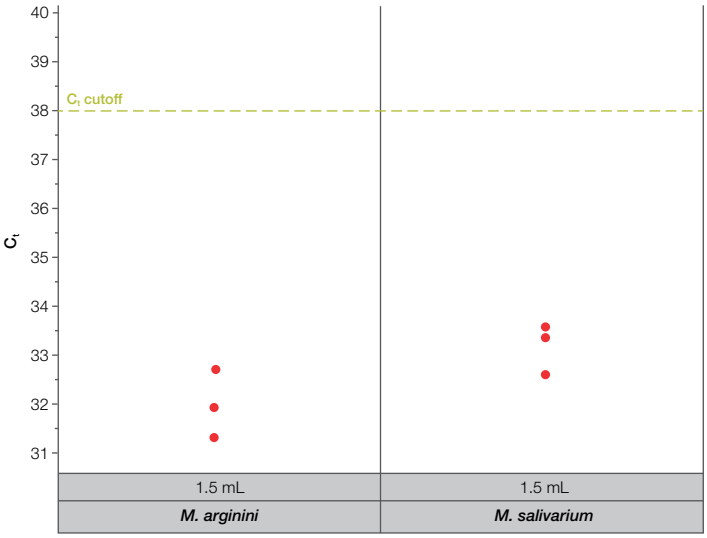

Figure 4. C_t values of *Mycoplasma* species spiked into cryopreservation medium and assayed using automatic extraction. The scatterplot shows the C_t values for gDNA of the noted species spiked into the medium containing 10^6 T cells at 10 GC/mL and processed using the automated PrepSEQ Express workflow followed by ethanol precipitation. The dotted line represents the C_t cutoff of the assay.

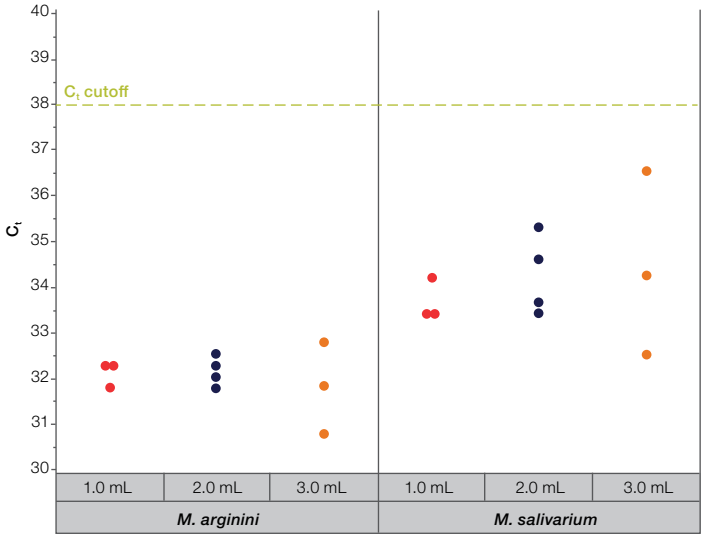

Figure 5. C_t values of *Mycoplasma* species spiked into cryopreservation medium and assayed using manual extraction. The scatterplot shows the C_t values for gDNA of the noted species spiked into the medium containing 10^6 T cells at 10 GC/mL and processed using the PrepSEQ manual workflow followed by ethanol precipitation. The dotted line represents the C_t cutoff of the assay.

Table 5. Comprehensive results of the MycoSEQ Plus Detection Kit with either the PrepSEQ manual or automated workflow with samples spiked into cryopreservation medium.

Species/target	GC /mL test sample	Sample prep method*	Sample volume (mL)	FAM dye mean C_t	S.D.** of FAM dye C_t	CV† (%) of FAM dye C_t	No. replicates	Detection rate (%)
M. arginini	10	ME	1	32.1	0.3	0.9	3	100
		ME	2	32.1	0.3	0.8	4	100
		ME	3	31.8	1.0	3.2	3	100
		AE	1.5	32.0	0.7	2.2	3	100
M. salivarium	10	ME	1	33.7	0.4	1.3	3	100
		ME	2	34.2	0.9	2.6	4	100
		ME	3	34.4	2.0	5.8	3	100
		AE	1.5	33.2	0.5	1.5	3	100
DPC/PEC‡	10	ME	1	35.5	0.8	2.4	3	100
		ME	2	34.5	0.3	1.0	4	100
		ME	3	34.7	1.6	4.7	3	100
		AE	1.5	35.4	1.1	3.2	3	100

* AE: automated extraction, ME: manual extraction.
** S.D.: standard deviation.
† CV: coefficient of variation.
‡ PEC: positive extraction control.

Figures 6 and 7 show the detection of all species, tested at 10 GC/mL in all 3 different types of matrices, as evidence of the consistent and robust detection that the MycoSEQ Plus assay enables in various matrices.

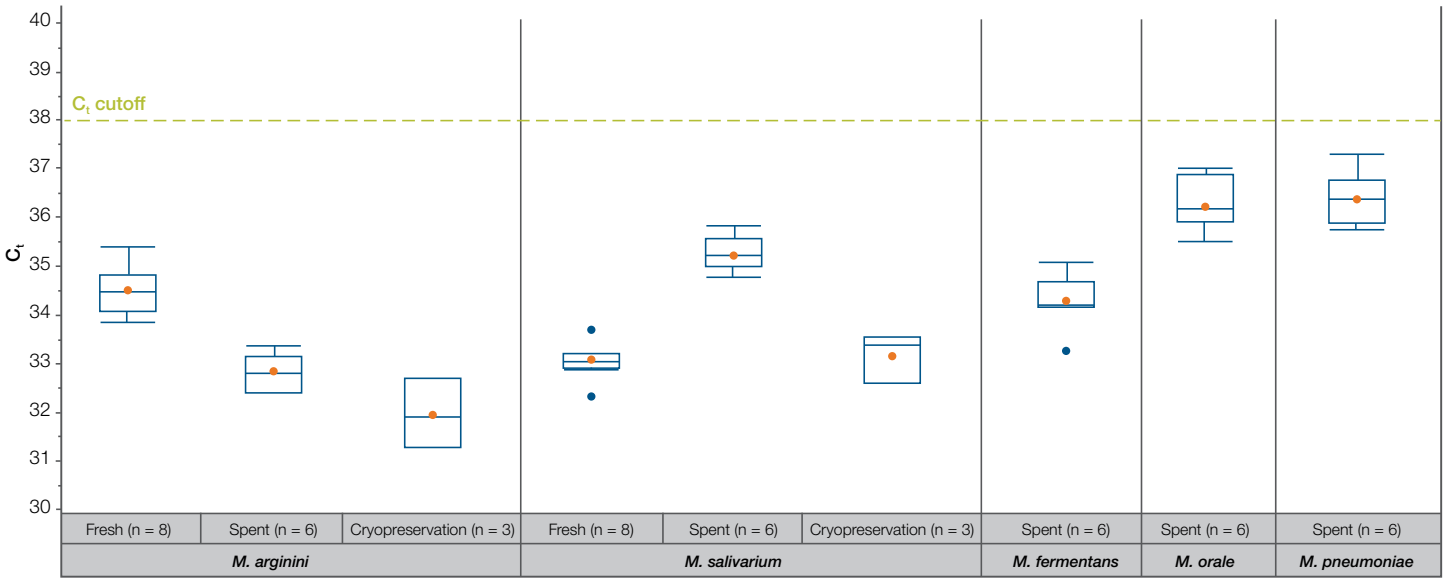

Figure 6. *Mycoplasma* species detected in 3 different matrices. The box plots show the C_t values for gDNA of the noted species spiked into fresh CTS, spent T cell, and cryopreservation media containing 10^6 T cells at 10 GC/mL and processed using the automated PrepSEQ Express workflow followed by ethanol precipitation. The dotted line represents the C_t cutoff of the assay.

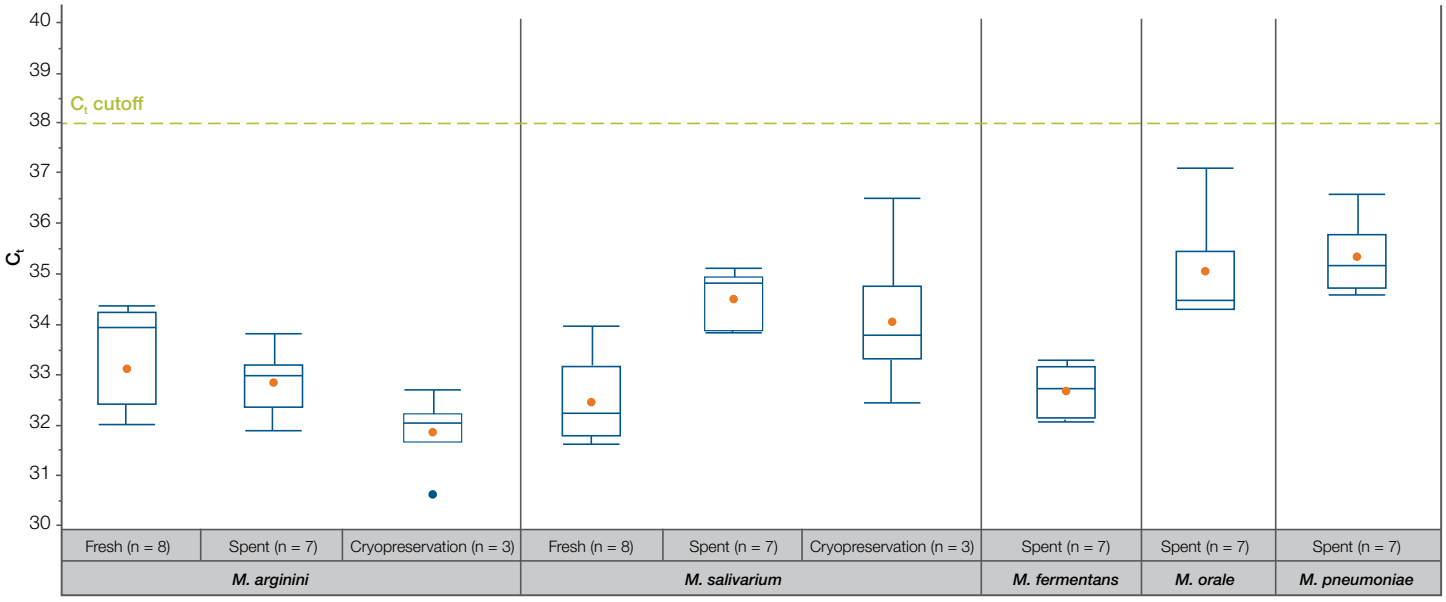

Figure 7. *Mycoplasma* species detected in 3 different matrices. The box plots show the C_t values for gDNA of the noted species spiked into fresh CTS, spent T cell, and cryopreservation media containing 10^6 T cells at 10 GC/mL and processed using the PrepSEQ manual workflow followed by ethanol precipitation. The dotted line represents the C_t cutoff of the assay.

Discussion

This application note details a method that should facilitate validation of the MycoSEQ Plus assay per the sensitivity guidance detailed in Ph. Eur. 2.6.7, Mycoplasmas [1]. This guidance specifies 10 CFU or genome copy equivalents per milliliter of test sample for nucleic acid tests. Our method uses as little as 1 mL of starting test sample.

The results demonstrate the comprehensive workflows for MycoSEQ Plus Mycoplasma Detection Kit, the manual workflow of the PrepSEQ Nucleic Acid Sample Preparation Kit and the automated workflow of the PrepSEQ Express Nucleic Acid Extraction Kit, and their seamless integration with AccuSEQ Real-Time PCR Detection Software.

Performance of the MycoSEQ Plus kit and the described method was evaluated using low-volume samples comprising representative T cell–containing matrices, including fresh CTS medium, spent T cell medium, and cryopreservation medium. The data show highly sensitive detection of gDNA from key *Mycoplasma* species (*M. arginini*, *M. salivarium*, *M. fermentans*, *M. orale*, and *M. pneumoniae*) in sample volumes of 1–3 mL.

Conclusion

An important feature of the MycoSEQ Plus system is the high sensitivity of the kit. Leveraging TaqMan chemistry, the assay is designed to accurately detect a wide range of *Mycoplasma* species, helping to ensure reliable results. This is crucial in maintaining the integrity of cell cultures, biologics, and other sensitive materials, as mycoplasma contamination can have detrimental effects on cell growth, product quality, and experimental outcomes.

Here we aimed to highlight the sensitivity of the MycoSEQ Plus Mycoplasma Detection Kit in the context of testing low-volume samples. Furthermore, the quick turnaround time enables faster decision-making, facilitating timely lot-release testing and reducing time to patient infusion for these advanced medicinal products. This is particularly valuable in the rapidly evolving field of cell therapy, where speed and efficiency are paramount. By providing a detailed overview of the materials, methods, and results, we aim to help enable manufacturers with valuable insights to enhance the safety, quality, and regulatory compliance of their cell therapy product.

References

1. European Pharmacopoeia 11.0, 2.6.7 Mycoplasmas.
2. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research (April 2008). Guidance for FDA reviewers and sponsors: content and review of chemistry, manufacturing, and control (CMC) information for human somatic cell therapy investigational new drug applications (INDs). **fda.gov/media/73624/download**

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