Pharma analytics

Sensitive and specific detection of mycoplasma DNA in low volume samples

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Abstract

Purpose: Leverage the Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection Kit within a cell therapy production workflow.

Methods: This protocol follows the MycoSEQ Plus mycoplasma detection system for test samples where input volume is limited. With this method, the equivalent of 1 mL of test sample is analyzed in the qPCR reaction. This leverages the sensitivity guidance of 10 colony forming units (CFU) or genome copy equivalents per mL of test sample.

Results: Samples were processed by either Applied Biosystems™ PrepSEQ™ manual or automated express workflows, followed by alcohol precipitation. PCR was performed on the Applied Biosystems™ QuantStudio™ 5 Real-time PCR system.

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.

Materials and methods

Sample preparation

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.

Test method(s)

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.

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

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. Thermal cycling run parameters are outlined in figure 1. 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.

Data analysis

The samples were processed by either PrepSEQ manual or automated PrepSEQ Express workflows, followed by alcohol 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.

Five different species and the MycoSEQ Plus DPC at 10 GC/mL were spiked into spent T cell medium with 10^6 T cells, and 2 species 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.

Results

Figure 1. Default qPCR cycling conditions in AccuSEQ software

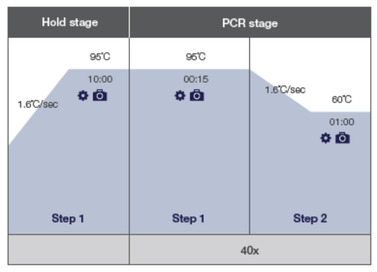
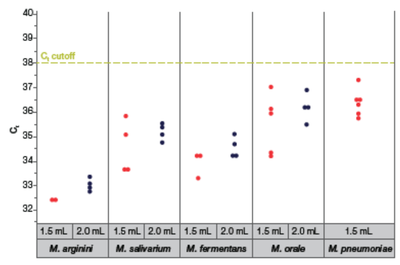
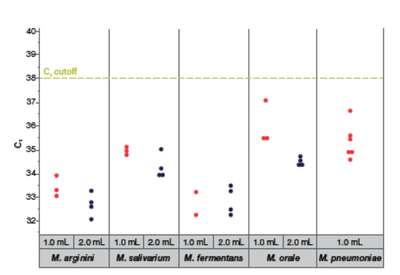


Figure 2. Ct values of *Mycoplasma* species spiked into spent medium and assayed using automated extraction.



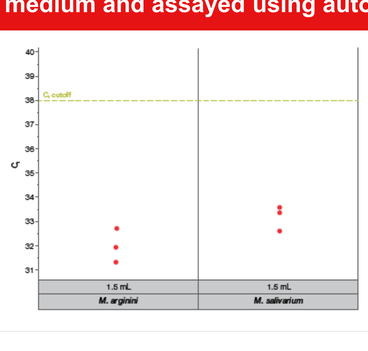
The scatterplot shows the cycle threshold (Ct) values for gDNA of the noted species spiked into spent medium containing 10^6 T cells at 10 GC/mL and processed using the automated PrepSEQ Express workflow followed by alcohol precipitation. The dotted line represents the Ct cutoff of the assay.

Figure 3. Ct values of *Mycoplasma* species spiked into spent medium and assayed using manual extraction.



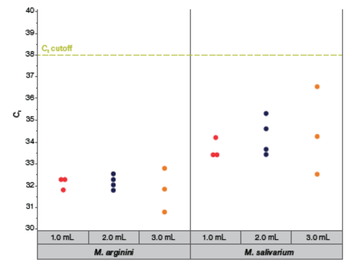
The scatterplot shows the threshold cycle (Ct) values for gDNA of the noted species spiked into spent medium containing 10^6 T cells at 10 GC/mL and processed using the PrepSEQ manual workflow followed by alcohol precipitation. The dotted line represents the Ct cutoff of the assay.

Figure 4. Ct values of *Mycoplasma* species spiked into cryopreservation medium and assayed using automated extraction



The scatterplot shows the Ct values for gDNA of the noted species spiked into cryopreservation medium containing 10^6 T cells at 10 GC/mL and processed using automated PrepSEQ Express workflow, followed by alcohol precipitation. The dotted line represents the Ct cutoff of the assay.

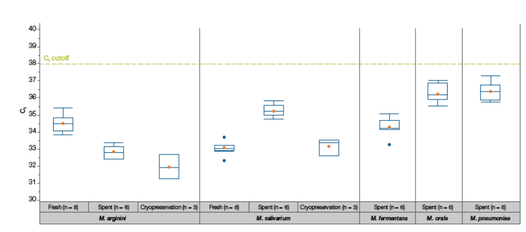
Figure 5. Ct values of *Mycoplasma* species spiked into cryopreservation medium and assayed using manual extraction



The scatterplot shows the Ct values for gDNA of the noted species spiked into cryopreservation medium containing 10^6 T cells at 10 GC/mL and processed using the PrepSEQ manual workflow followed by alcohol precipitation. The dotted line represents the Ct cutoff of the assay.

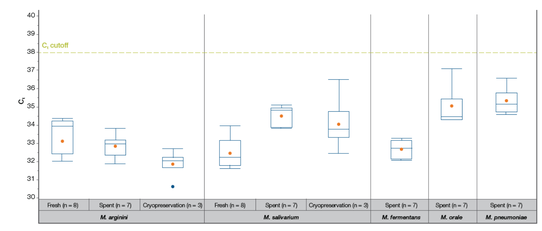
Results (cont'd)

Figure 6. *Mycoplasma* species detected in 3 different matrices using an automated workflow



The box plots show the Ct 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 alcohol precipitation. The dotted line represents the Ct cutoff of the assay.

Figure 7. *Mycoplasma* species detected in 3 different matrices using a manual worflow.



The box plots show the Ct values for gDNA of the noted species spiked into fresh CTS, spent T cell media, and cryopreservation media containing 10^6 T cells at 10 GC/mL and processed using the PrepSEQ manual workflow followed by alcohol precipitation. The dotted line represents the Ct cutoff of the assay.

Conclusions

The MycoSEQ Plus assay is designed to detect mycoplasma contamination in accordance with Ph. Eur. 2.6.7, Mycoplasmas guidelines for nucleic acid tests, achieving sensitivity down to 10 CFU or genome copy equivalent/mL. The method is optimized for low-volume samples, requiring only 1 mL, which is critical for advanced medicinal products. It integrates the MycoSEQ Plus Mycoplasma Detection Kit with workflows, either manual or automated, using PrepSEQ kits. Alcohol precipitation is used to concentrate DNA from extracted samples, enhancing sensitivity. This robust system demonstrates high sensitivity and reliable detection across diverse sample types, including various T cell media and cryopreservation medium.

The MycoSEQ Plus system's performance facilitates the high sensitivity and reliability necessary for the detection of mycoplasma species, which is vital for the quality and safety of biologics and cell-based therapies. Using TaqMan chemistry, the assay detects key mycoplasma species, maintaining the integrity of cell cultures and other sensitive materials. This makes it suitable for the fast-paced field of cell therapy, facilitating quicker decision-making and timely product release.

Key takeaways

- MycoSEQ Plus assay meets relevant regulatory guidelines with high sensitivity.
- Effective for 1 and 1.5 mL low-volume samples (using ME or AME, respectively), supporting advanced medicinal products
- Compatible with both manual and automated workflows for flexible use.
- Facilitates reliable detection of various mycoplasma species, critical for determining the safety and quality of cell therapies and biologics.

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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