Improving time to result: Leveraging rapid mycoplasma detection and sterility testing in cell therapy manufacturing

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Abstract

Biotherapies, and especially cell therapy products, are required to be tested for sterility and mycoplasma. Developing an analytical strategy to test for sterility and mycoplasma can be daunting. There are many variables to consider, including complex matrices with high mammalian cell density and detection of non-viable microbes. Which type of analytical testing can work in an early stage of therapeutic development and then be scaled to meet the challenges later in the production process?

Leveraging rapid sterility as well as mycoplasma qPCR-based detection techniques can help provide confidence in the final product by helping to detect potential contamination earlier in the production process.

Introduction

The recently updated US FDA guidelines, which is applicable to genetically engineered cell products such as CAR-T cell therapies, recommends that Mycoplasma and adventitious agent testing should be conducted during the manufacturing stage when contamination is most likely to be detected, such as after pooling of cultures for harvest prior to cell washing. However, as is anticipated in the regulatory guidance, it is often the case that traditional testing methods cannot be used with cellular products due to the lengthy timeframes involved and the limited product shelf-life and vein-to-vein time available. Alternative methods that may be suitable for such products include rapid sterility tests and rapid PCR-based Mycoplasma tests. Any such rapid detection assays must offer adequate sensitivity and specificity.

Sterility testing guidance states that to help ensure product safety, all cell therapies, such as CAR T cells, should be free of viable contaminating microorganisms. Importantly, the final drug product cannot be sterilized by filtration or permanently sterilized (steam), as the cells must remain fully viable and functional. Therefore, product safety is further supported using sterility testing per the United States Pharmacopeia (USP) <71>, or an appropriately validated alternative test method per USP <1223>.

Materials and methods

Rapid mycoplasma testing

Here we demonstrate low volume mycoplasma sample testing using the Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection system with complex T cell-containing media samples commonly used in gene and cell therapy bioproduction workflows.

Testing of T cell-containing media samples

Mycoplasma genomic DNA was spiked into each sample (10 genome copies per mL (GC/mL), depending on the sample extraction method and sample volume; see Table 1). Samples were processed with both Applied Biosystems™ PrepSEQ™ Manual and Automated Express workflow followed by ethanol precipitation steps to concentrate final eluate.

Sample preparation

We designed and executed experiments to demonstrate the MycoSEQ Plus Mycoplasma Detection Kit assay performance at workflow LOD (i.e., 10 GC/mL) in low volume samples comprised of complex T cell-containing matrices, including fresh CTS media, spent T cell media, or lot-release media, allowing highly sensitive detection of key mycoplasma species listed in Pharmacopeia (*M. arginini, M. salivarium, M. orale, M. fermentans, and M. pneumoniae*) and MycoSEQ™ Plus Discriminatory positive control included in the kit in sample volumes as low as 1 mL. Low volume samples were processed with PrepSEQ Manual and Automated workflows using the Applied Biosystems™ PrepSEQ™ Express Nucleic Acid Extraction Kit followed by ethanol precipitation steps to concentrate the final eluate.

Similarly, 10 mL sample volumes were also tested in manual and automated with Applied Biosystems™ King Fisher™ Flex workflows.

Sample processing

Low volume (i.e.,1 - 3 mL) samples of spent T cell media (or fresh CTS media or lot-release media) containing 10^6 T-cells were preprocessed by centrifugation at 1,000 x *g* to pellet the mammalian cells. The supernatant containing mycoplasma was transferred to a new tube and centrifuged at 16,000 x *g* to pellet the mycoplasma. The supernatant was discarded, and mycoplasma pellet retained on ice. The mammalian cell pellet was 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 supernatant was transferred to the mycoplasma pellet and resuspended followed by DNA template spike-in at workflow LOD respective to low volume sample (i.e.: 10 GC/mL).

Automated extraction

The Applied Biosystems™ AutoMate Express™ Nucleic Acid Extraction System enables automated recovery of mycoplasma DNA from complex samples. In each of the sample tubes provided in the PrepSEQ Express Nucleic Acid Extraction Kit, 300 µL of sample with or without mycoplasma DNA was added. Samples were loaded into the Applied Biosystems™ AutoMate Express system for DNA extraction using the PrepSEQ Express kit protocol with 30 minutes of Proteinase K lysis and eluted into 100 µL of elution buffer.

Manual extraction

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

Ethanol Precipitation

PrepSEQ sample eluates underwent ethanol precipitation steps to concentrate the PrepSEQ eluates. Optimized volume ratios of isopropanol were added to sample eluates with 0.3M Sodium Acetate salts followed by centrifugation at 16,000 x *g* for 15 mins at 4° C. The supernatant was removed, and the DNA pellet washed with 70% ethanol followed by centrifugation at 16,000 x *g* for 5 mins at 4° C. The supernatant was removed allowing the DNA pellet to air-dry for 5 mins and redissolved in appropriate volume (see Table 1) of PrepSEQ elution buffer by incubating at 55° C for 30 mins with gentle shaking (RPM ~ 750 RPM).

MycoSEQ Plus Mycoplasma Detection Assay

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

qPCR setup and run

Samples for each of the reaction mixtures were prepared as shown in Table 2. The MycoSEQ Plus Mycoplasma Detection kit contains 2x qPCR Master Mix Plus, 10x qPCR assay mix, DNA control (positive control), and Negative control water (no-template control). The 96-well 0.1 mL plate was loaded into an QuantStudio 5 Real-Time PCR System with 96-well 0.1 mL block and running the Applied Biosystems™ AccuSEQ™ Real-Time PCR Detection Software v3.2. The following thermal cycling run parameters were used for the qPCR run): 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 discriminatory positive control (DPC), and the internal positive control (IPC), respectively.

Table 1. DNA spike-in concentrations and volumes for sample extraction workflows

Sample preparation workflow	Starting sample volume	DNA spike-in at workflow LOD (10 GC/mL)	EtOH precipitation final elution volume	DNA Detected in PCR reaction (GC/Rxn)	Number of Rxns per extraction
AME	3 mL	30 GC	36 µL	6.7 GC	3
AME	2 mL	20 GC	24 µL	6.7 GC	2
AME	1.5 mL	15 GC	12 µL	10 GC	1
Manual	3 mL	30 GC	36 µL	10 GC	3
Manual	2 mL	20 GC	24 µL	10 GC	2
Manual	1 mL	10 GC	12 µL	10 GC	1

Table 2. 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 qPCR 10X Assay Mix	3	33
Negative Control Water	N/A	N/A
Total	**30**	**198**

USP<71> species testing

The species tested included *Aspergillus brasiliensis, Bacillus subtilis, Candida albicans, Clostridium sporogenes, Pseudomonas aeruginosa, and Staphylococcoccus aureus.* Organisms were spiked into either dilution buffer or a cell culture matrix with 10^6 Jurkat cells and cryopreservation media. Spiked samples were pelleted at 15,000 x *g*, and supernatants were removed prior to extraction with one of two possible extraction kits. Eluted samples were then tested using the rapid sterility testing kit and QuantStudio™ 5 Real-Time PCR System with Applied Biosystems™ AccuSEQ™ software.

Pelleting study

A discriminatory positive control, included in the kit, was diluted in DNA dilution buffer and then added to a cell culture matrix prior to centrifuging at 15,000 x *g*. The cell culture matrix contained 10^6 Jurkat cells and cryopreservation media. Following the centrifugation step, the pellet underwent DNA extraction. A separate control set was also tested, with addition of DPC to the matrix without centrifugation. Extracted samples were then tested using the qPCR kit and run using the QuantStudio 5 system with the AccuSEQ software.

Results

Low volume testing

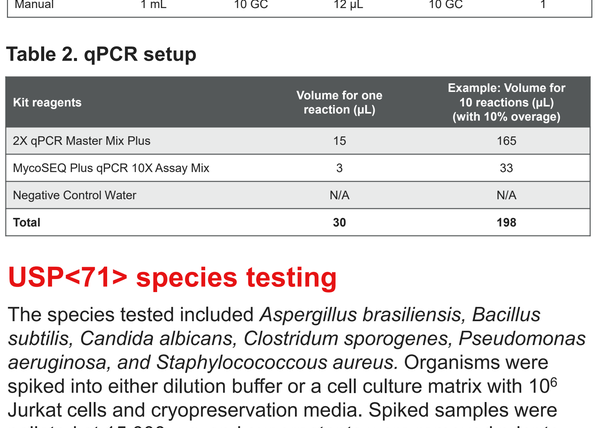

Figure 1: Detecting species in T-cell spent media

The plot demonstrates the resulting Ct values of detected Mycoplasma species spiked into spent T cell media containing 10^6 cells and processed following automation and ethanol precipitation.

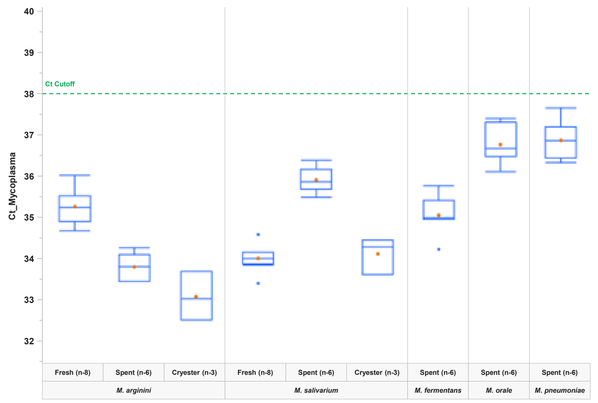

Figure 2: Detecting Mycoplasma species at LOD in low-volume samples

The plots indicate the consistent detection of Mycoplasma species in three different matrices: fresh media, spent T cell media, and final lot release media, containing 10^6 T cells at LOD (10 GC/mL) and processed following automated sample prep.

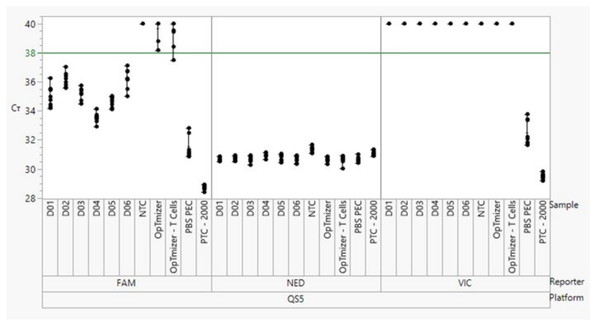

Figure 3: Detecting Mycoplasma species at LOD in 10 mL sample volumes

The plots demonstrate the positive detection of Mycoplasma species above the Ct value within the samples containing T cells, as tested using the QuantStudio 5 system following a manual extraction protocol.

Label	Species	FAM		VIC		NED		Detection (≥23/24)
		Mean Ct	Ct SD	Mean Ct	Ct SD	Mean Ct	Ct SD	
D01	*Mycoplasma orale*	35.03	0.69	40.00	0.00	30.69	0.12	24/24 (100%)
D02	*Mycoplasma salivarium*	36.08	0.51	40.00	0.00	30.71	0.14	24/24 (100%)
D03	*Acholeoplasma laidlawii*	35.15	0.43	40.00	0.00	30.65	0.20	24/24 (100%)
D04	*Mycoplasma arginini*	33.53	0.33	40.00	0.00	30.93	0.14	24/24 (100%)
D05	*Mycoplasma fermentans*	34.66	0.34	40.00	0.00	30.80	0.20	24/24 (100%)
D06	*Mycoplasma hyorhinis*	36.26	0.67	40.00	0.00	30.68	0.21	24/24 (100%)
NTC		40.00	0.00	40.00	0.00	31.37	0.19	N/A
OpTmizer		39.66	0.69	40.00	0.00	30.66	0.17	N/A
OpTmizer – T Cells		39.43	0.90	40.00	0.00	30.64	0.26	N/A
PBS PEC(200 copies)		31.58	0.79	32.48	0.81	30.67	0.17	N/A
PTC - 2000		28.74	0.14	29.48	0.15	31.06	0.12	N/A

Table 3: Result summary for samples tested using a manual workflow on the QuantStudio 5.

The table above illustrates the consistent detection of various Mycoplasma species in the 10mL samples.

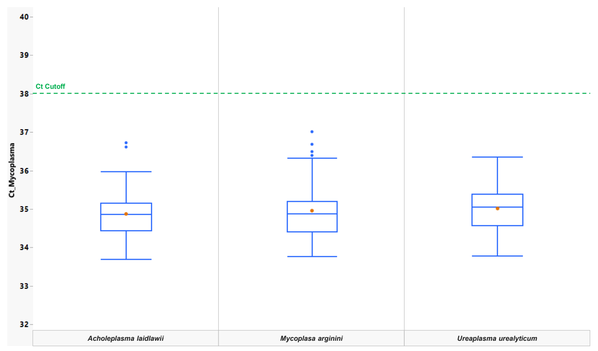

Figure 4: Boxplot of mycoplasma species spiked into spent T cell media containing 10^6 T cells at workflow LOD (10 GC/mL) and processed by Kingfisher Flex workflow.

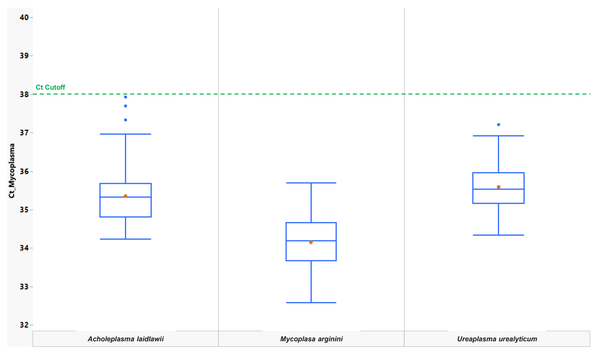

Figure 5: Boxplot of mycoplasma species spiked into spent T cell media containing 10^6 T cells at workflow LOD (10 GC/mL) and processed by PrepSEQ Manual extraction workflow.

Live bacteria and fungi detection

All 6 USP <71> species, were detected at an LOD of 10-99 CFU. The workflow has shown compatibility with cell culture matrices containing 10^6 mammalian cells.

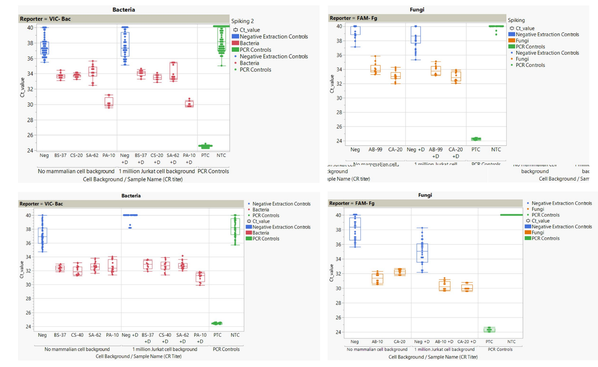

Figure 3. Ct values for bacteria and fungi samples spiked at LOD prior to extraction using one of two possible kits. AB: *Aspergillus brasiliensis*, CA: *Candida albicans*, BS: *Bacillus subtilis*, CS: *Clostridium sporogenes*, PA: *Pseudonomas aeruginosa*, SA: *Staphylococcous aureus*. Numbers following abbreviated species names refer to the titer (LOD in CFU) that was spiked in prior to sample preparation. Samples noted with "+D" included a matrix containing 10^6 Jurkat cells in cryopreservation media.

Pelleting study

When compared against samples that did not undergo centrifugation, samples which were centrifuged prior to sample preparation displayed an 8 Ct increase in mean Ct values. This indicates a removal of >99% of floating DNA during centrifugation. This further suggests that including a centrifugation step at the start of sample preparation helps enable the removal of a large proportion of floating DNA, reducing the potential for detection of DNA from lysed, non-viable microbes in test samples.

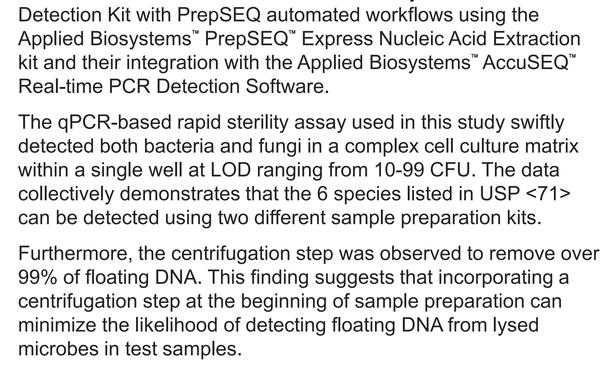

Figure 4. Effect of centrifugation on detection of floating DNA. DPC without Cen.: positive control-spiked samples without centrifugation; DPC with Cen.: positive-control-spiked samples that were centrifuged at 15,000 x *g* for 5 minutes prior to sample preparation. Negative samples refer to samples without positive control spike.

Conclusions:

The MycoSEQ Plus assay performance at LOD (10 GC/mL) is demonstrated in low volume samples comprised of complex T cell-containing matrices, including spent T-cell media, and final formulation media, as well as 10 mL sample volumes. We demonstrated that the assay was able to detect key species listed in several pharmacopeia (e.g., *M. arginini, M. salivarium, M. fermentans, M. orale, and M. pneumoniae*) and MycoSEQ Plus Discriminatory Positive Control included in the kit in sample volumes as low as 1 mL.

We also demonstrated the workflow of the MycoSEQ Plus Detection Kit with PrepSEQ automated workflows using the Applied Biosystems™ PrepSEQ™ Express Nucleic Acid Extraction kit and their integration with the Applied Biosystems™ AccuSEQ™ Real-time PCR Detection Software.

The qPCR-based rapid sterility assay used in this study swiftly detected both bacteria and fungi in a complex cell culture matrix within a single well at LOD ranging from 10-99 CFU. The data collectively demonstrates that the 6 species listed in USP <71> can be detected using two different sample preparation kits.

Furthermore, the centrifugation step was observed to remove over 99% of floating DNA. This finding suggests that incorporating a centrifugation step at the beginning of sample preparation can minimize the likelihood of detecting floating DNA from lysed microbes in test samples.

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