Pharma analytics

Mycoplasma testing in monoclonal antibody production

Implementing the MycoSEQ Plus mycoplasma detection system throughout various stages of a CHO cell–based bioproduction process

Introduction

Mycoplasma contamination poses a significant risk to bioproduction processes that utilize mammalian cell culture or animal-derived materials. The presence of mycoplasmas can alter cellular metabolism and other processes, potentially altering subsequent biotherapy product quality and introducing additional patient risk. Small, lacking a cell wall, and slow-growing, mycoplasmas are challenging to both detect and remove. Complex workflows are at greater risk of contamination. In the case of monoclonal antibody (mAb) or other large-scale therapies, it can be quite costly to remove the affected batch and disinfect the facilities. Assessment for mycoplasma risk and development of an appropriate testing program can help prevent costly and time-consuming investigations due to mycoplasma contamination.

Two methods are most often used to monitor mycoplasmas in bioproduction materials and processes: the culture-based growth test and the PCR-based nucleic acid test (NAT). The culture-based growth test, described in United States Pharmacopeia (USP) chapter <63>, involves culturing a sample in growth conditions favorable to mycoplasmas, then assessing for mycoplasma growth after 28 days. The NAT test uses sample-derived nucleic acid as a template for enzymatic amplification. This amplification product is measured by fluorescence-based methods to determine if mycoplasmas are present. For more information on NAT testing, please refer to European Pharmacopoeia (Ph. Eur.) chapter <2.6.7>.

The Applied Biosystems™ MycoSEQ™ Plus Mycoplasma Detection Kit is a PCR-based method for detecting the presence of mycoplasmas in raw materials, cell banks, in-process samples, and lot-release samples. The MycoSEQ Plus system was designed and tested to meet or exceed regulatory requirements listed in several pharmacopoeias (i.e., USP, Ph. Eur., and Japanese Pharmacopoeia) for lot-release performance, including specificity, sensitivity, and robustness. Unlike the growth-based test, live mycoplasmas are not needed to run this test, and actionable results may be obtained in less than one day.

Here we demonstrate mycoplasma testing using the MycoSEQ Plus system with media samples from a CHO cell–based mAb production workflow. Test samples were taken during in-process bioreactor growth and bulk harvest lot release.

Testing of in-process bioreactor samples

Samples and sample preparation

CHO cell culture samples were collected on day 10 of bioreactor growth (Figure 1). Samples were used undiluted at 300 µL per sample type.

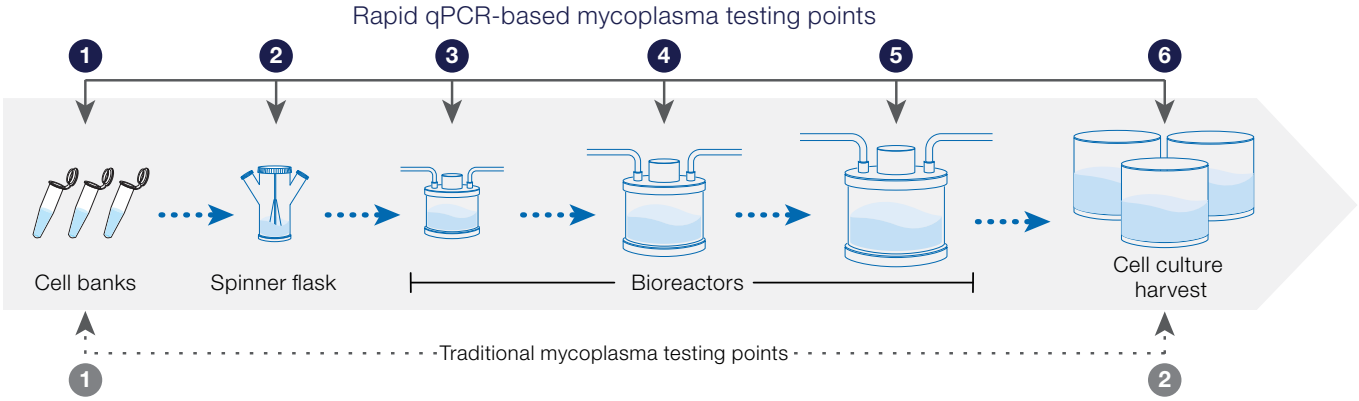

Figure 1. Cell culture manufacturing process for cells taken from a cell bank (step 1) until cell culture harvest (step 6). In-process samples of CHO cells were collected from the bioreactor noted at step 5.

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Mycoplasma nucleic acid was spiked into each sample (either 500 or 1,000 genome copies (GC), depending on the sample extraction method; Table 1). Nucleic acid was also spiked into 1X PBS for use as a positive extraction control. Samples were processed in parallel using either automated or manual nucleic acid extraction.

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 Applied Biosystems™ PrepSEQ™ *Express* Nucleic Acid Extraction Kit, 300 µL of CHO cell in-process sample (or 1X PBS) 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 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 CHO cell in-process sample (or 1X PBS) 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.

qPCR setup and run

Samples for each of the reaction mixtures were prepared as shown in Table 2. The MycoSEQ Plus kit contains a master mix, an assay mix, DNA control (positive control), and nuclease-free water (no-template control).

The 96-well plate was loaded into an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System running the Applied Biosystems™ AccuSEQ™ Real-Time PCR Detection Software v3.2. The following thermal parameters were used for the qPCR run (Figure 3): 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 types and concentrations for sample extraction workflows.

		Automated workflow		Manual workflow	
	DNA type	Initial GC spike-in	Final GC/reaction in qPCR	Initial GC spike-in	Final GC/reaction in qPCR
Mycoplasma arginini	Genomic	7,500	500	10,000	1,000
Acholeplasma laidlawii	Genomic	7,500	500	10,000	1,000
Discriminatory positive control (DPC)	Plasmid	7,500	500	10,000	1,000

Table 2. qPCR setup.

Components	Volume per reaction (µL)
2X qPCR Master Mix Plus	15
MycoSEQ Plus qPCR 10X Assay Mix	3
Nuclease-free water	2
Template	10
Total	**30**

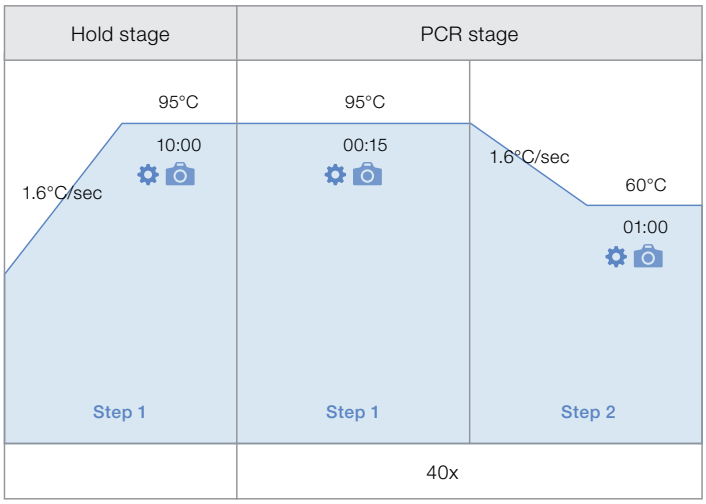


Figure 3. Default qPCR cycling conditions in AccuSEQ software v3.2.

Results

After DNA extraction on the AutoMate *Express* system, the analysis using AccuSEQ software v3.2 showed that the CHO cell in-process samples spiked with mycoplasma DNA yielded passing C_t values (detected in the FAM dye channel) and 100% detection rates (Table 3, Figure 4). The C_t values were comparable to those of the positive extraction controls in 1X PBS, indicating that sample extraction was robust. Samples that were not spiked with DNA template did not give any background C_t signal (i.e., "undetermined" signal), which demonstrated no false positive signals from the matrix itself.

Table 3. Detection of species in CHO cell in-process sample matrix (and PBS extraction controls) processed on the AutoMate *Express* system.

Matrix	Species	Copies per reaction	Mean of C_t (FAM dye)	CV (%) of C_t (FAM dye)	Number of replicates	Detection rate
CHO cell in-process sample matrix	*M. arginini*	500	29.03	0.16	9/9	100%
	A. laidlawii	500	29.89	0.25	9/9	100%
	DPC	500	31.11	0.45	9/9	100%
	None	0	Undetermined	–	0/9	0%
PBS	*M. arginini*	500	29.15	1.08	3/3	100%
	A. laidlawii	500	30.02	0.41	3/3	100%
	DPC	500	31.22	0.34	3/3	100%
	None	0	Undetermined	–	0/3	0%

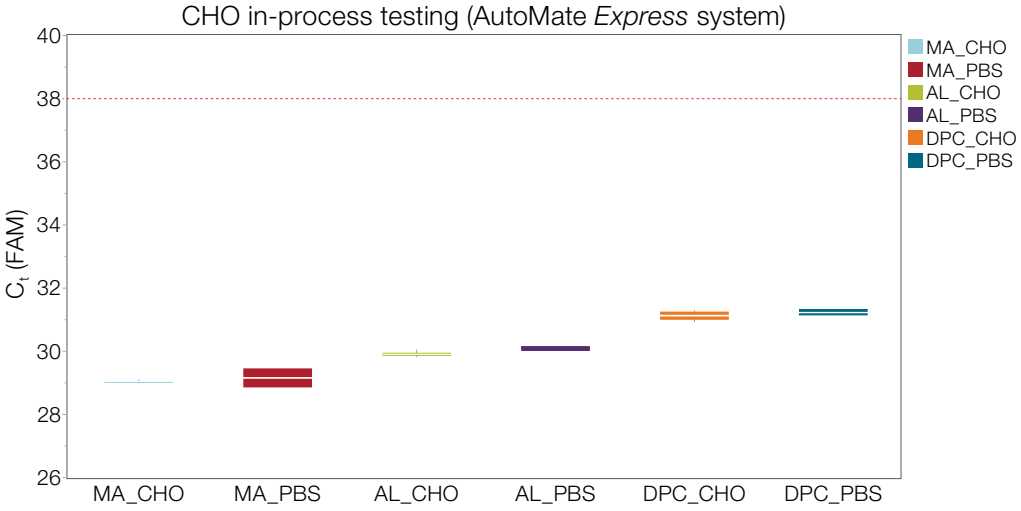


Figure 4. C_t values of CHO cell in-process test samples and PBS controls spiked with *M. arginini* (MA), *A. laidlawii* (AL), or DPC and processed on the AutoMate *Express* system.
All C_t values are well below the cutoff of 38 (dashed red line).

Similar results were observed with the alternative method of extracting samples manually. The CHO in-process samples spiked with mycoplasma DNA yielded passing C_t values and 100% detection rates (Table 4, Figure 5).

Testing of bulk harvest samples

Sample preparation

The bulk harvest media were received as bioreactor contents that had been preprocessed in one of two ways. In the first method, bioreactor contents were passed through a 2-stage depth filter (removing cells, cell debris, and some host cell proteins) and then through a 0.2 µm filter. In the second, the bioreactor contents were centrifuged (thus removing cells), then passed through a single-stage depth filter (removing cell debris and some host cell proteins), and then through a 0.2 µm filter. Because of this preprocessing, the bulk harvest samples were spiked with 2×10^5 CHO cells to reflect the composition of an unprocessed bulk harvest sample. Bulk harvest media containing CHO cells were then spiked with DNA templates representing 15 species of mycoplasma, at the limit of detection (LOD), 10 GC/reaction.

Automated extraction and qPCR analysis

Bulk harvest media samples were processed using the large-scale automated protocol of the PrepSEQ *Express* Nucleic Acid Extraction Kit (i.e., supernatant only; option 1 in the user guide) on the AutoMate *Express* system. Reaction mixtures were prepared with components included in the MycoSEQ Plus Mycoplasma Detection Kit, and run on the QuantStudio 5 Real-Time PCR System with AccuSEQ software v3.2.

Table 4. Detection of species in CHO cell in-process sample matrix (and PBS extraction controls) processed through a manual sample extraction workflow.

Matrix	Species	Copies per Reaction	Mean of C_t (FAM dye)	CV (%) of C_t (FAM dye)	Number of replicates	Detection rate
CHO cell in-process sample matrix	*M. arginini*	1,000	28.66	0.39	9/9	100%
	A. laidlawii	1,000	29.48	0.26	9/9	100%
	DPC	1,000	32.39	0.59	9/9	100%
	None	0	Undetermined	–	0/9	0%
PBS	*M. arginini*	1,000	31.46	0.78	3/3	100%
	A. laidlawii	1,000	31.57	2.92	3/3	100%
	DPC	1,000	33.57	1.04	3/3	100%
	None	0	Undetermined	–	0/3	0%

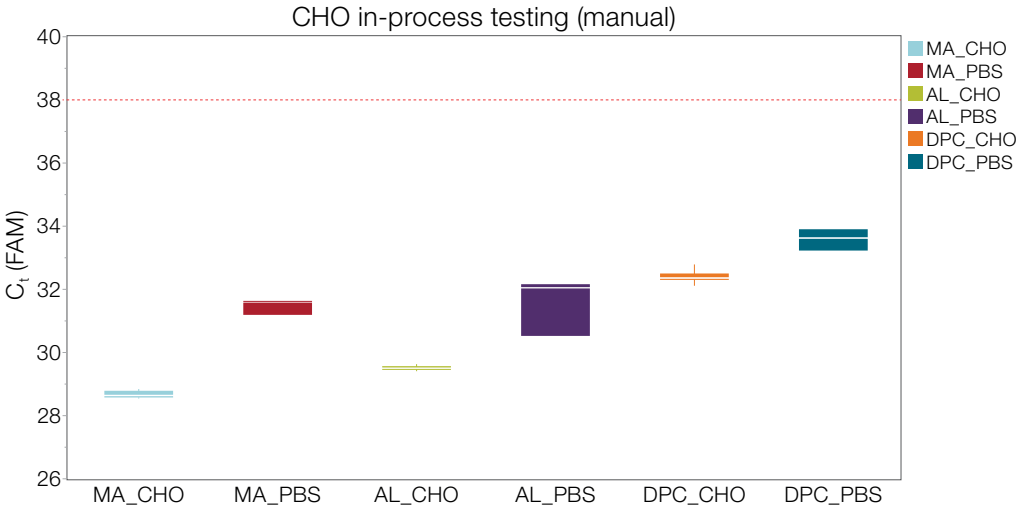


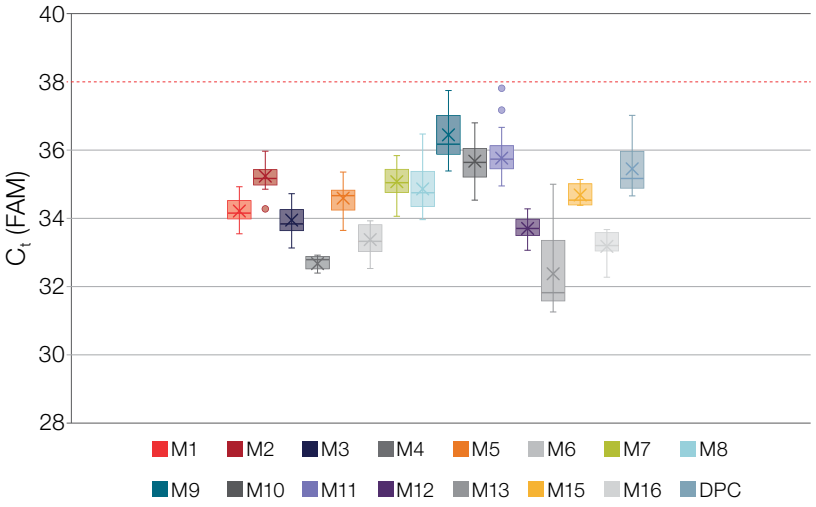
Figure 5. C_t values of CHO cell in-process test samples and PBS controls spiked with *M. arginini* (MA), *A. laidlawii* (AL), or DPC and then processed manually. All C_t values are well below the cutoff of 38 (dashed red line).

Results

High sensitivity for the 15 species (10 species listed in pharmacopoeias and 5 additional common species) was demonstrated in the complex background of the bulk harvest media containing CHO cells (Table 5, Figure 6). All species were detected at a rate of 100%. The results confirmed assay performance in bulk harvest media, which potentially include high background interference.

Table 5. Detection of species in CHO cell bulk harvest media processed on the AutoMate *Express* system.

Species	Copies per reaction	Mean ± SD of C_t (FAM dye)	CV (%) of C_t (FAM dye)	Number of replicates	Detection rate
Mycoplasma hominis	10	34.20 ± 0.41	1.20	8	100%
Mycoplasma orale	10	35.23 ± 0.39	1.11	24	100%
Mycoplasma salivarium	10	33.92 ± 0.42	1.23	24	100%
Mycoplasma pirum	10	32.70 ± 0.20	0.61	8	100%
Mycoplasma pneumoniae	10	34.57 ± 0.47	1.35	24	100%
Mycoplasma synoviae	10	33.35 ± 0.40	1.21	24	100%
Mycoplasma arginini	10	35.06 ± 0.43	1.22	24	100%
Mycoplasma fermentans	10	34.84 ± 0.64	1.84	24	100%
Acholeplasma laidlawii	10	36.44 ± 0.70	1.92	24	100%
Spiroplasma citrii	10	35.66 ± 0.59	1.66	24	100%
Mycoplasma hyorhinis	10	35.86 ± 0.73	2.04	24	100%
Mycoplasma gallisepticum	10	33.69 ± 0.34	1.01	24	100%
Mycoplasma mycoides	10	32.35 ± 1.33	4.11	8	100%
Mycoplasma haemohominis	10	34.67 ± 0.31	0.88	8	100%
Mesoplasma tabanidae	10	33.17 ± 0.44	1.33	8	100%



Species ID	Organism
M1	*M. hominis*
M2	*M. orale*
M3	*M. salivarium*
M4	*M. pirum*
M5	*M. pneumoniae*
M6	*M. synoviae*
M7	*M. arginini*
M8	*M. fermentans*
M9	*A. laidlawii*
M10	*M. citrii*
M11	*M. hyorhinis*
M12	*M. gallisepticum*
M13	*M. mycoides*
M15	*M. haemohominis*
M16	*M. tabanidae*

Figure 6. C_t values of CHO cell bulk harvest media spiked with different species and processed on the AutoMate *Express* system. DNA from fifteen species at LOD (10 copies/reaction) were spiked into CHO cell bulk harvest media, extracted, and analyzed on the QuantStudio 5 Real-Time PCR System. The DPC (PCR positive control) is also presented to qualify the run. The top and bottom sides of the boxplot are the upper and lower quartiles. The box covers the interquartile interval, where 50% of the data are found. The vertical line that splits the box in two is the median and the "x" is the mean. The dashed red line at a C_t (for the FAM dye) of 38 represents the cutoff for the assay.

Conclusions

Biotherapies using processes susceptible to mycoplasma contamination must be tested and found free of mycoplasmas prior to release. Longer, complex, or expensive processes may also benefit from including testing at different steps of production, depending on a relevant risk assessment and control strategy plan. Examples of possible testing points include cell banks, in-process samples, and bulk harvest material.

In this application note, the capability of the MycoSEQ Plus system to detect mycoplasmas was demonstrated using samples related to common testing points in a mAb production process. Not all sample testing requires the same level of sensitivity. This study demonstrates a method for testing a very small amount of material that may be used for analyzing in-process samples, which do not usually require the most sensitive level of detection. However, regulatory guidance clearly defines LOD performance needed to confirm bulk harvest for lot release. As per EP chapter <2.6.7>, LOD is defined as 10 CFU/mL, or in this case for a NAT-based method, 10 GC/mL of sample. Data in this study confirm that the MycoSEQ Plus Mycoplasma Detection Kit, with suitable sample preparation, can provide accurate results on the presence (or absence) of 10 GC/mL mycoplasma DNA spiked into a related sample matrix, therefore meeting regulatory guidance. This sensitive level of detection was achieved using genomic DNA from various notable *Mycoplasma* species and either automated or manual sample preparation protocols.

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