Challenge
Conducting Mycoplasma testing for cell-based therapeutics with high specificity and in a timely manner

Solution
Implementation of the Microsart® ATMP Mycoplasma Assay and the Microsart® Calibration Reagent by Sartorius Stedim Biotech GmbH on the qTOWERiris 96 and 384 (Analytik Jena GmbH+Co. KG)

Intended audience
Scientists, researchers, and technicians in biotech and pharmaceutical industry that use qPCR for mycoplasma detection, ensuring cell culture integrity and product safety

Detection of cell wall lacking bacterial species using the real-time PCR cycler qTOWERiris

Introduction

The detection of Mycoplasma, Acholeplasma or Spiroplasma (Mollicutes) in cell cultures plays a major role. This is particularly important in the pharmaceutical industry for biological substances obtained from cell cultures, such as Advance Therapy Medicinal Products (ATMP).
Assays like the Microsart® ATMP Mycoplasma (Sartorius Stedim Biotech GmbH) follow the standards outlined in the European Pharmacopoeia (EP) 2.6.7 „Mycoplasmas“.[1]

Beyond detecting mollicutes, quantifying genomic copy numbers is often imperative. For this purpose, the Microsart® Calibration Reagent from Sartorius can also be employed. A real-time PCR cycler with high sensitivity and accuracy is also essential. Analytik Jena's qTOWERiris series meets both criteria and allows upscaling with the qTOWERiris 384 for high-throughput PCR.

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Materials and Methods

Chemicals and consumables

- Microsart® Calibration Reagent (Sartorius Stedim Biotech GmbH), cat: SMB95-2026
- Microsart® ATMP Mycoplasma (Sartorius Stedim Biotech GmbH), cat: SMB95-1003
- 96-well PCR Plate (0.2 mL; LP), full-skirted, white, (Analytik Jena GmbH+Co. KG), cat: 844-70038-S
- 384-well PCR Plate, full-skirted, white, suitable for automation (Analytik Jena GmbH+Co. KG), cat: 844-70039-0
- Optical sealing foil, (Analytik Jena GmbH+Co. KG), cat: 844-70046-0

Instrumentation

The real-time PCR thermocycler qTOWERiris 96 and qTOWERiris 384 (Analytik Jena GmbH+Co. KG) including:

- Color module 1, blue (455 nm / 515 nm) was used for FAM™ dye
- Color module 4, orange (580 nm / 620 nm) was used for ROX™ dye

Following the instructions for the Microsart® Calibration Reagent, a dilution series of the calibration reagent was pipetted to create a calibration curve. The Mycoplasma mix, the internal control DNA and the positive control DNA were rehydrated according to the instructions. A reaction mix was prepared for the corresponding number of reactions. The six dilution levels, the positive control, and the no target control (NTC) were pipetted in duplicate reactions. In this experiment, the internal control served as an amplification control.
For the qTOWERiris 96 and qTOWERiris 384, 15 µL of reaction mix and 10 µL of sample, positive control and NTC were transferred to the corresponding plates for the different formats.
The protocol on both units was defined according to Table 1, selecting color modules 1 and 4 to capture fluorescence, with FAM™ as the target and ROX™ as the internal control.

Table 1: Temperature and time protocol.

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	3 min	8 °C/s
2	45	Denaturation	95 °C	30 sec	8 °C/s
		Annealing	55 °C	30 sec	5.5 °C/s
		Elongation*	60 °C	45 sec	8 °C/s

*Data acquisition with color module 1 and 4 for qTOWERiris series, gain 5.0.

Results and Discussion

1. qTOWERiris 96

Utilizing a 10 µL sample volume, the target concentrations of the standards were assigned in the qTOWERiris software, following the guidelines in the Calibration Reagent manual. The dilution of the standards showed excellent amplification results (Figure 1) and generated a standard curve with appropriate R^2 and PCR efficiency values (Figure 2), analyzed using the "Absolute Quantification" tool in qPCRsoft software. Additionally, the samples demonstrated high homogeneity with a standard deviation below 0.2 (Table 2). If the positive control is considered as an unknown sample, its concentration was determined to be 4175 genome copies.

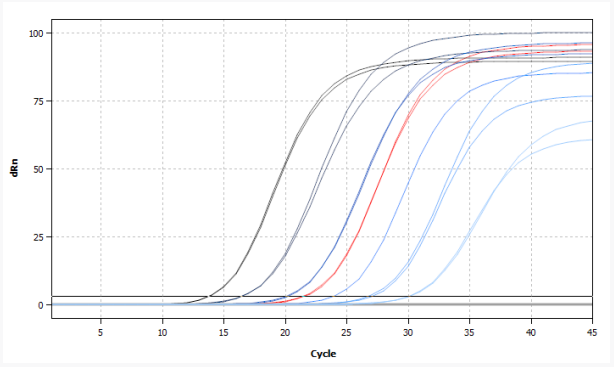


Figure 1: Amplification curves of the target for FAM™, with an automatic threshold of 3.224.

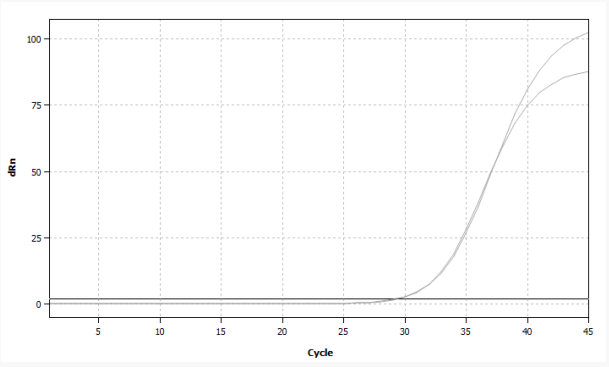
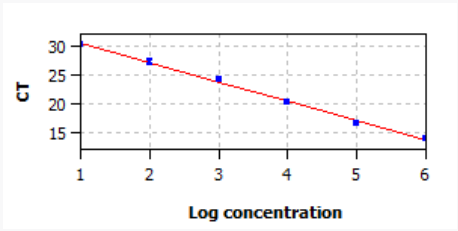


Figure 3: Amplification curve for the NTC for ROX™ as internal control with an automatic threshold of 3.224.



	FAM
R^2	0.99789
Slope	-3.33
Offset	33.66
PCR efficiency	1

Figure 2: Results of the standard curve.

Table 2: Mean Ct, standard deviation, and concentration of all samples with threshold 3.224.

Target	Mean Ct	Standard deviation	Concentration [genome copies per reaction]
Standard 1	13.92	0.02	1,000,000
Standard 2	16.60	0.01	100,000
Standard 3	20.22	0.08	10,000
Standard 4	24.06	0.00	1000
Standard 5	27.05	0.18	100
Standard 6	30.19	0.00	10
Positive control	21.60	0.09	4175.15
No target control (NTC)	No Ct	-	-

The assay can be considered valid because the internal amplification control showed a signal in the ROX channel for the NTC and not for the target in the FAM channel (Figure 3). However, the internal control does not play a role for the evaluation if there was a positive signal detected in the target channel.

2. qTOWERiris 384

After successfully demonstrating the performance of the Microsart® ATMP Mycoplasma Assay on the qTOWERiris 96, the experiment was repeated on the qTOWERiris 384. The same dilution, positive control, and sample volume were used. The results of the standard curve showed similarly appropriate values in the qTOWERiris 384 as in the qTOWERiris 96 (Figure 4). The PCR efficiency and the coefficient of determination are within an optimal range (Figure 5). Table 3 also shows the Ct values and the concentrations. The standards from the qTOWERiris 384 assay differed by two Ct values compared to the qTOWERiris 96 assay, possibly due to differences in the threshold values.

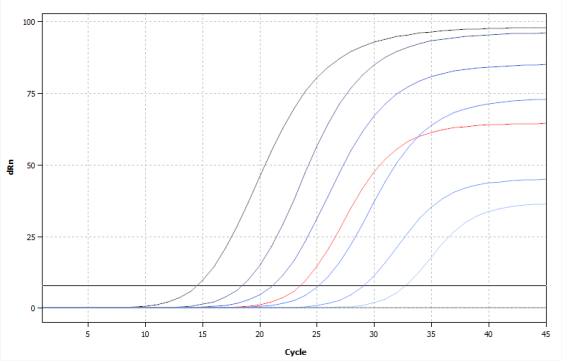


Figure 4: Amplification curves of the target for FAM™ with an automatic threshold of 7.808.

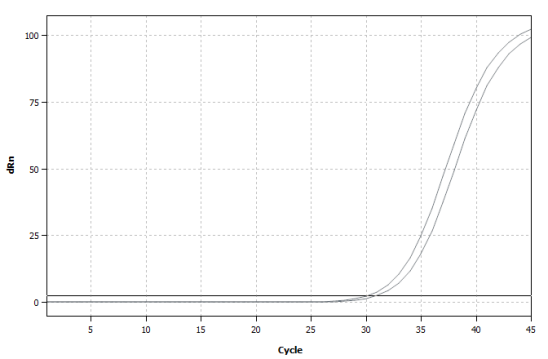
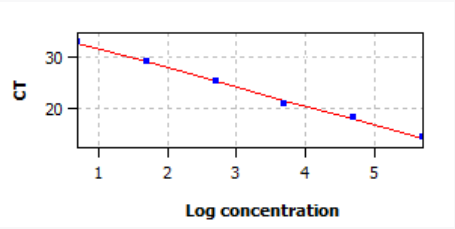


Figure 6: Amplification curve for the NTC for ROX as internal control.



	FAM
R^2	0.99754
Slope	-3.64
Offset	35.17
PCR efficiency	0.88

Figure 5: Results of the calibration curve.

Table 3: Ct values, and concentration of all samples with threshold 7.808.

Target	Mean Ct	Concentration [genome copies per reaction]
Standard 1	14.51	1000,000
Standard 2	18.44	100,000
Standard 3	21.10	10,000
Standard 4	25.19	1000
Standard 5	29.14	100
Standard 6	32.76	10
Positive control	23.54	4034.43
No target control (NTC)	No Ct	-

The test on the qTOWERiris 384 can be considered valid, since a signal in the target channel for FAM™ was generated for all standards and the positive controls while the NTC showed no amplification for FAM™ but for the internal control for ROX (Figure 6).

Conclusion

The detection of Mollicutes (Mycoplasma, Acholeplasma, and Spiroplasma) contamination is crucial for those working with cell cultures, particularly in the field of Advanced Therapy Medicinal Products (ATMP). Assays like the Microsart® ATMP Mycoplasma from Sartorius Stedim Biotech GmbH enable the detection of such contaminants, and when combined with the Microsart® Calibration Reagent, providing the feasibility for quantification. The results here demonstrate the excellent performance of the assay and Calibration Reagent with the qTOWERiris, showing adequate PCR efficiency and homogeneity between replicates. High-throughput applications are feasible with the qTOWERiris 384, as supported by the available data. The combined use of qTOWERiris 96 and 384, offered by Analytik Jena Gmbh+Co. KG, coupled with Microsart® ATMP Mycoplasma from Sartorius Stedim Biotech GmbH, represents a robust solution for Mollicutes detection, in alignment with the European Pharmacopoeia standards, particularly in quality assurance.

Figure 7: qTOWERiris Series.

Recommended device configuration

Table 4: Overview of required equipment and recommended accessories and consumables.

Article	Article number	Description
qTOWERiris 96 incl. Color Module 1	844-00853-x*	Real-time PCR system designed in the 96-well format, operable via PC, customizable with up to 6 color modules. Available in 100 V, 115 V, and 230 V version.
qTOWERiris 384 incl. Color Module 1	844-00858-x*	Real-time PCR system designed in the 384-well format, operable via PC, customizable with up to 6 color modules. Available in 100 V, 115 V, and 230 V version.
Color Module 4 for qTOWERiris series	844-00823-0	Color module for the excitation and emission of fluorescence dyes like ROX™, TexasRed®, Cy3.5® or ATTO590.
96-well PCR plate	844-70038-S	96 well full-skirted PCR plate, volume 0.2 mL, low profile, white
384-well PCR plate	844-70039-0	384 well full-skirted PCR plate, white, suitable for automation
Optical sealing foil	844-70046-0	Highly transparent, adhesive sealing foils suitable for standard 96 or 384 well (q)PCR plates (79 x 142 mm, peelable, free of DNA, ATP, PCR inhibitors and endotoxins, free of DNases and RNases).

*x=2 for 230 V, x=4 for 115 V and x=5 for 100 V

References

[1] European Pharmacopoeia – Chapter 2.6.27 Microbiological Examination of cell-based Preparations revised: https://www.gmp-compliance.org/gmp-news/revision-of-chapter-2-6-7-of-the-european-pharmacopoeia-published-for-comment

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