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Cyclus® Rapid Mycoplasma

Mycoplasma Detection in ATMP-Relevant Cell Culture Matrices

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Abstract

Rapid mycoplasma detection is essential for quality control of cell culture-based biopharmaceuticals and advanced therapy medicinal products (ATMPs), particularly under the revised EP 2.6.7 (12.2), which endorses nucleic acid amplification techniques (NAT) as alternatives to traditional culture methods. This Application Note evaluates the Cyclus® Rapid Mycoplasma Workflow, designed to meet EP-aligned sensitivity requirements (≤10 CFU/mL or <100 GC/mL) while providing robust process control. The workflow combines Cyclus® Bead Extraction for nucleic acid isolation, the Cyclus® RT-qPCR Mycoplasma detection kit, and Cyclus® Mycoplasma Standards for qualification and validation. Performance was assessed in three matrices: Cell culture medium, and HEK cell culture at 5×10^6 and 1×10^7 cells/mL, representing typical and highly challenging conditions. Across all matrices, multiple mycoplasma species were reliably detected at EP-relevant levels, with internal controls confirming efficient extraction and negligible inhibition. Additional robustness testing on different qPCR platforms in a high-density HEK matrix demonstrated consistent assay performance and no platform-specific limitations. These results show that the Cyclus® Rapid Mycoplasma portfolio offers a flexible, sensitive, and EP 2.6.7 (12.2)-aligned NAT solution for routine mycoplasma testing and product-specific validation in diverse QC laboratory settings.

Find out more: www.sartorius.com/en/products/microbiological-testing/rapid-mycoplasma-detection

Introduction & Objective

Rapid mycoplasma testing has become an essential element in the quality control of cell culture–based biopharmaceuticals and ATMPs, particularly under the revised EP 2.6.7 (12.2) framework. The new chapter recognizes nucleic acid amplification techniques (NAT) as equivalent to culture-based methods, but links this acceptance to clearly defined performance, control and validation criteria. In practice, this requires NAT assays to maintain high sensitivity (≤ 10 CFU/mL or < 100 GC/mL), perform reliably in relevant product matrices and demonstrate robust process control from extraction through amplification.

The Cyclus® Rapid Mycoplasma portfolio was developed to address these requirements as an integrated solution. It combines the Cyclus® Bead Extraction kit for magnetic bead–based nucleic acid isolation with the Cyclus® RT-qPCR Mycoplasma kit for DNA/RNA detection and the new Cyclus® 10 CFU and Cyclus® 100 GC Mycoplasma Standards for EP-aligned validation and process control.

In this Application Note, we describe an initial performance evaluation of the Cyclus® workflow in three test scenarios:

- **Scenario 1:** Cyclus® performance in RNase-free cell culture medium
- **Scenario 2:** Cyclus® performance in a cell matrix containing 5 × 10^6 HEK cells/mL
- **Scenario 3:** Cyclus® performance in a high-density cell matrix containing 1 × 10^7 HEK cells/mL

Scenario 1 is designed to verify Cyclus® Bead Extraction and Cyclus® RT-qPCR Mycoplasma performance in a simple, RNase-free matrix using the standard protocol for RNase-free cell culture media.

To challenge the Cyclus® workflow in a realistic cell culture matrix, two experiments were performed using HEK cell suspensions. In Scenario 2 a matrix is used containing 5 × 10^6 HEK cells/mL, representing typical cell densities used in production processes or ATMP manufacturing. In Scenario 3 a more demanding matrix was used with 1 × 10^7 HEK cells/mL, simulating high-density cultures or worst-case sample conditions.

In all experiments, the focus was on demonstrating reliable detection of different mycoplasma species at EP-relevant levels while maintaining appropriate control performance.

Material

Cyclus® Rapid Mycoplasma Detection portfolio used:

- **Cyclus® Bead Extraction** kit (manual workflow used; optional KingFisher™ Flex compatible for automation)
- **Cyclus® RT-qPCR Mycoplasma** kit (RT-qPCR with TaqMan® probes, FAM™ channel for target, HEX™/VIC™ for internal control)
- **Cyclus® 10 CFU Acholeplasma laidlawii** (inactivated CFU)
- **Cyclus® 100 GC Mycoplasma orale**
- **Cyclus® 100 GC Mycoplasma fermentans** (dPCR quantified genomic copies)

The chosen species represent typical EP/JP/USP-relevant mycoplasma contaminants, with *Acholeplasma laidlawii* being a frequently used reference organism for method verification in cell culture–based manufacturing.

Experimental design

All extractions were performed manually according to the Cyclus® Bead Extraction instructions for the respective matrix type (RNase-free vs. RNase-containing). For each condition, the following layout was used:

- Each sample was extracted in duplicate.
- Each extract was analyzed in duplicate by Cyclus® RT-qPCR.

This resulted in a total of four RT-qPCR reactions per condition (2 extractions × 2 PCR replicates). A schematic sample overview for Scenario 1-3 is shown in Table 1.

Table 1: Schematic sample overview for Scenario 1-3

Samples
Matrix & 100 GC/mL *Mycoplasma orale*
Matrix & 100 GC/mL *Mycoplasma fermentans*
Matrix & 10 CFU/mL *Acholeplasma laidlawii*
Controls
NEC: Negative extraction control using only Matrix without spike
NTC: PCR negative control using only PCR-grade water
PC: PCR positive control using supplied PC template

Methods

Scenario 1: Extraction protocol for RNase-free matrices
In Scenario 1 DMEM was used as matrix. A cell-free medium like DMEM does typically not contain any RNases. Because of the RNase absence, the standards were rehydrated using the protocol for RNase-free matrices (see Instructions for use Cyclus® 10 CFU and Cyclus® 100 GC "Protocol 1").

As instructed, DMEM is used directly for rehydration of the standards (e.g. Cyclus® 10 CFU or Cyclus® 100 GC). Next, the Cyclus® Bead Extraction is used to isolate and purify all nucleic acids. First, the sample is lysed with Proteinase K and lysis buffer to release mycoplasma nucleic acids. A binding mix containing magnetic beads is then added so that DNA and RNA from the inactivated CFU or the genomic copy standard binds to the beads. After magnetic separation, the supernatant is discarded, and the beads are washed in successive wash buffers to remove matrix components and PCR inhibitors. Following a short drying step, nucleic acids are eluted with elution buffer, and the clear eluate is transferred to a fresh tube for direct use in the Cyclus® RT-qPCR Mycoplasma assay. In nuclease free matrices, no additional protective steps are required because the integrity of the nucleic acids in the Cyclus® 10 CFU and Cyclus® 100 GC standards is preserved throughout the process.

Scenario 2 & 3: Adapted extraction protocol for RNase-containing matrices
Upon cell lysis, intracellular RNases are released and can degrade mycoplasma rRNA, potentially affecting RT-qPCR sensitivity. To mitigate this, the Cyclus® 100 GC Standards and mycoplasma nucleic acids were processed using the RNase-containing protocol (see Instructions for use Cyclus® 10 CFU and Cyclus® 100 GC "Protocol 2").

Cyclus® 10 CFU or Cyclus® 100 GC are rehydrated in protective lysis buffer before they are added to the RNase-containing matrix. This way, the protocol is adapted to rapidly inactivate endogenous nucleases released from the cells and to stabilize mycoplasma nucleic acids from the inactivated CFU or genomic copy standards before degradation can occur. This ensures that the rRNA from the Cyclus® 10 CFU or Cyclus® 100 GC remains intact and is not compromised by nuclease activity. As a result, the Cyclus® RT-qPCR assay can still exploit its RNA and DNA detection capability, even in high cell density or otherwise challenging matrices, and reliably demonstrate EP aligned sensitivity in product-like samples.

Next, the Cyclus® Bead Extraction is used to isolate and purify all nucleic acids as described above for Scenario 1.

Result Summary

The controls perform in all scenarios as expected. In the FAM™ target channel, NEC and NTC remain negative, while the positive controls perform within the expected Cq range (Table 2). In all controls the HEX™/VIC™ control channel showed consistent results (Table 3). Thus, the tested matrices have no inhibitory effects on PCR performance.

All mycoplasma species were reliably detected in all three scenarios in all replicates at the targeted spike levels (Table 2). Internal control amplification confirmed successful extraction and absence of relevant inhibition (data not shown) indicating that the adapted RNase-containing protocol efficiently removed inhibitors and preserved nucleic acids for Scenarios 2 & 3.

The experiments across three matrices – RNase-free medium, 5×10^6 HEK cells/mL and 1×10^7 HEK cells/mL – show that the Cyclus® Rapid Mycoplasma portfolio provides a robust and flexible workflow for NAT-based mycoplasma detection under EP 2.6.7 (12.2).

Sample	Extraction Replicate	PCR Replicate	Scenario 1 DMEM	Scenario 2 5 M HEK cells/mL	Scenario 3 10 M HEK cells/mL
PC	-	1	26.08	26.39	26.24
PC	-	2	25.89	24.95	25.92
PC	-	3	26.20	25.55	26.00
PC	-	4	26.55	25.69	26.25
NTC	-	1	Undetermined	Undetermined	Undetermined
NTC	-	2	Undetermined	Undetermined	Undetermined
NEC (just Matrix)	A	1	Undetermined	Undetermined	Undetermined
NEC (just Matrix)	A	2	Undetermined	Undetermined	Undetermined
Matrix & 100 GC/mL *Mycoplasma orale*	A	1	32.71	32.46	32.60
Matrix & 100 GC/mL *Mycoplasma orale*	A	2	33.37	32.04	32.85
Matrix & 100 GC/mL *Mycoplasma orale*	B	1	31.38	31.81	31.72
Matrix & 100 GC/mL *Mycoplasma orale*	B	2	31.36	31.72	32.25
Matrix & 100 GC/mL *Mycoplasma fermentans*	A	1	29.00	30.37	30.59
Matrix & 100 GC/mL *Mycoplasma fermentans*	A	2	29.46	30.18	30.38
Matrix & 100 GC/mL *Mycoplasma fermentans*	B	1	29.12	30.27	31.19
Matrix & 100 GC/mL *Mycoplasma fermentans*	B	2	29.92	29.66	30.70
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	A	1	30.25	32.34	31.88
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	A	2	30.52	31.67	31.89
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	B	1	31.01	32.57	31.97
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	B	2	30.96	32.49	32.07

Table 2: RT-qPCR results (Cq values of FAM™ target channel) of all scenarios. All results were generated using the Quantstudio 5 qPCR cycler.

			Scenario 1 DMEM	Scenario 2 5 M HEK cells/mL	Scenario 3 10 M HEK cells/mL
NTC	-	1	30.18	29.66	33.23
NTC	-	2	32.83	26.4	32.02
NEC (just Matrix)	A	1	30.42	35.5	33.14
NEC (just Matrix)	A	2	30.41	29.05	33.89

Table 3: RT-qPCR results (Cq values of HEX™/VIC™ control channel) of the negative controls of all scenarios. All results were generated using the Quantstudio 5 qPCR cycler.

Robustness across different PCR instruments

Objective

This experiment was designed to evaluate the robustness of the Cyclus® Rapid Mycoplasma workflow across different real-time PCR instruments. For Scenario 1-3 a Quantstudio 5 system from Thermo Fisher was used. While Scenarios 1–3 focused on matrix effects (RNase-free medium and HEK cell suspensions at 5×10^6 and 1×10^7 cells/mL), this experiment assessed whether the Cyclus® Bead Extraction kit and Cyclus® RT-qPCR Mycoplasma kit deliver comparable performance on a different qPCR platform, a qTOWERiris from Analytik Jena, using a challenging, cell-containing matrix.

Experimental setup

A HEK cell matrix at 1×10^7 cells/mL, as described in Scenario 3, was used as a worst-case model. Samples were spiked with a panel of Cyclus® 100 GC mycoplasma standards *(Mycoplasma orale, Mycoplasma fermentans, Mycoplasma pneumoniae, Mycoplasma salivarium).* Extraction was performed manually with the Cyclus® Bead Extraction protocol for RNase-containing matrices, as described for Scenario 2 & 3 to preserve nucleic acids in the presence of cellular RNases.

As in the previous scenarios, each condition was extracted in duplicate, and each extract was analyzed in duplicate RT-qPCR reactions (4 reactions per condition) using the Analytik Jena qTOWERiris

Results

All replicates of the spiked samples were reliably detected in the FAM™ target channel. Internal control signals (HEX™/VIC™ channel) were consistently within the expected Cq range on the qTOWERiris, indicating robust extraction and minimal PCR inhibition regardless of the instrument used. Negative controls (NEC and NTC) remained free of target signals on the qTOWERiris, and positive controls fell within predefined acceptance limits. To conclude, no platform-specific false positives or loss of sensitivity were observed.

Conclusion

The results of this experiment demonstrate that the Cyclus® Rapid Mycoplasma workflow – comprising Cyclus® Bead Extraction, Cyclus® RT-qPCR Mycoplasma and Cyclus® Standards – performs robustly across different qPCR platforms, even in a high-density HEK cell matrix. This cross-platform robustness supports the use of the Cyclus® portfolio in diverse laboratory environments and simplifies technology transfer and method implementation under the revised EP 2.6.7 (12.2) framework.

Sample	Extraction Replicate	PCR Replicate	FAM™ Target Channel	HEX™/VIC™ Control Channel
Positive Control	-	1	26.29	31.52
Positive Control	-	2	25.9	28.84
Positive Control	-	3	25.96	27.33
Positive Control	-	4	26.38	28.87
NTC	-	1	Undetermined	32.59
NTC	-	2	Undetermined	32.18
NEC (just Matrix)	A	1	Undetermined	30.48
NEC (just Matrix)	A	2	Undetermined	31.76
Matrix & 100 GC/mL *Mycoplasma orale*	A	1	33.58	31.14
Matrix & 100 GC/mL *Mycoplasma orale*	A	2	33.11	29.98
Matrix & 100 GC/mL *Mycoplasma orale*	B	1	32.45	27.14
Matrix & 100 GC/mL *Mycoplasma orale*	B	2	30	28.88
Matrix & 100 GC/mL *Mycoplasma fermentans*	A	1	29.82	31.16
Matrix & 100 GC/mL *Mycoplasma fermentans*	A	2	30.26	28.32
Matrix & 100 GC/mL *Mycoplasma fermentans*	B	1	29.66	25.91
Matrix & 100 GC/mL *Mycoplasma fermentans*	B	2	29.41	28.55
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	A	1	31.59	37.39
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	A	2	31.74	30.3
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	B	1	31.24	28.85
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	B	2	33.45	27.19
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	A	1	31.06	32.66
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	A	2	31.08	26.27
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	B	1	32.97	27.08
Matrix & 10 CFU/mL *Acholeplasma laidlawii*	B	2	32.41	29.39

Table 4: RT-qPCR results (Cq values of FAM™ target channel & HEX™/VIC™ control channel) of the qTOWERiris using 1×10^7 HEK cells/mL as matrix.

Key observations

- Consistent detection of *Mycoplasma orale, Mycoplasma fermentans, Mycoplasma pneumoniae, Mycoplasma salivarium* and *Acholeplasma laidlawii* at EP-relevant GC and CFU levels in all tested matrices.
- Stable internal control performance, confirming efficient extraction and low inhibition in both medium and cell-containing samples.
- Negative NEC and NTC in all runs, supporting the contamination-mitigating design of the workflow (bead-based extraction, RT-qPCR, lyophilized reagents and defined controls).
- Suitability of Cyclus® 10 CFU and Cyclus® 100 GC Standards as external reference materials for sensitivity verification, matrix inhibition testing and EP-aligned validation concepts (including CFU/GC ratio).
- Comparable Cq values and identical qualitative results across different real-time PCR platforms in the high-density HEK matrix underline the robustness of the Cyclus® workflow for cross platform implementation and method transfer.

All extractions in this evaluation were performed manually; however, the Cyclus® Bead Extraction protocol is also compatible with automated magnetic particle processors such as the KingFisher™ Flex, enabling higher throughput laboratories to scale up the workflow without changing the core chemistry.

Conclusion

The data generated in this study demonstrate that the Cyclus® Bead Extraction kit, the Cyclus® RT-qPCR Mycoplasma kit and the new Cyclus® 10 CFU / Cyclus® 100 GC Mycoplasma Standards together form a powerful, EP 2.6.7 (12.2)-aligned solution for NAT-based mycoplasma testing.

The workflow performed robustly in both RNase-free media and HEK cell matrices up to 1×10^7 cells/mL, with reliable detection of all spiked mycoplasma species and satisfactory control results throughout.

These findings support the use of the Cyclus® Rapid Mycoplasma portfolio for:

- routine QC testing of cell culture–based products and ATMPs
- product-specific validation and risk-based method verification according to the revised EP 2.6.7 requirements.

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