Fast and sensitive mycoplasma detection workflow using RT-dPCR with same-day results

Mycoplasma detection

Mycoplasmas are common contaminants in biological products derived from cell lines in the biopharmaceutical industry. They are often introduced either from the source cell lines or during production. Reliable detection is critical to ensure product safety and regulatory compliance in advanced therapeutic manufacturing, where even low-level contamination can lead to significant delays or batch rejection.

Digital PCR (dPCR) is an effective method for detecting these mycoplasma contaminations in cell cultures and related biological products. Unlike traditional broth/agar culture methods, the QIAcuity RT-dPCR workflow is a fast, reproducible and highly sensitive alternative for mycoplasma detection. Viable mycoplasma cells have numerous rRNA copies. Thus, targeting both rRNA and DNA in this workflow increases detection sensitivity. This is particularly important for low-level contaminations, which can otherwise go undetected. The RT-based method is therefore more accurate for risk management and enables more reliable decision-making during manufacturing or batch release. This same-day, pharmacopeia-compliant workflow minimizes hands-on time while maintaining high reproducibility across complex matrices such as CHO cell suspensions and media.

The QIAcuity Mycoplasma Quant Kit is a highly sensitive RT-dPCR kit that detects rRNA and DNA, covering 127 mycoplasma species. The kit includes an internal control to prevent false negatives caused by PCR inhibitors or errors in RNA extraction and RT reactions. Besides, the associated QIAcuity Mycoplasma Standard CFU Kits contain 10 different Mycoplasma CFU Standards for validation and optional positive sensitivity controls.

When paired with the EZ2 Connect automated sample preparation platform, the workflow supports consistent, high-throughput nucleic acid purification with reduced operator variability, ideal for regulated environments.

Combined with a recommended sample prep method, the QIAcuity Mycoplasma Quant Kit enables a Nucleic Acid Technique (NAT) workflow for mycoplasma testing that is compliant with the European Pharmacopeia (EP; chapter 2.6.7), the US Pharmacopeia (USP; chapter <63>), and the Japanese Pharmacopeia (JP; chapter G3-14-170). The robust process eliminates time-consuming cultivation of mycoplasma. The workflow is particularly suited to sample types of varying purity (for example, cell bank samples, in-process samples, such as virus harvest and final lots/batches). This approach supports regulatory compliance while enabling faster and safer delivery of advanced therapeutics. ▷

QIAcuity Mycoplasma Quant Kit workflow is validated to meet pharmacopeia requirements

The Pharmacopeia-compliant NAT workflow using the QIAcuity Mycoplasma Quant Kit consists of three steps and can be conducted within one day. First, the sample is processed using the EZ2 Connect protocol with the EZ1&2® Virus Mini Kit v2.0 – Mycoplasma, an automated nucleic acid isolation method adapted for mycoplasma testing.

During extraction, the Mycoplasma Internal Control is spiked in as a process control. Then the QIAcuity Mycoplasma Quant Master Mix is prepared and dispensed into a preplate. Eluates are added, and the reaction mixture is transferred to a 26k Nanoplate. Finally, the nanoplate is placed into the QIAcuity Digital PCR instrument, and the RT-dPCR run is started (Figure 1). The results are analyzed using the QIAcuity Software Suite (Figure 2). This fast and easy-to-follow protocol supports same-day results, reducing the decision-making time in quality control workflows.

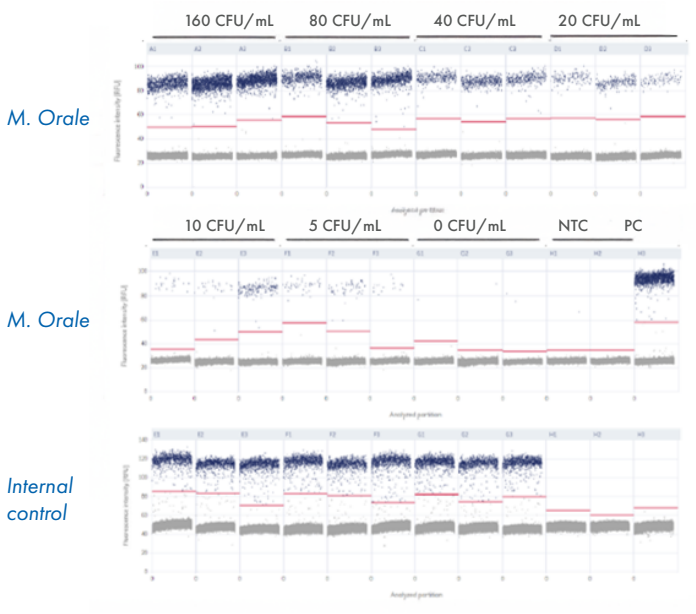

Figure 2. RT-dPCR 1D scatterplots of *M. orale* in DMEM +10% FCS and Internal Control Spike-in.

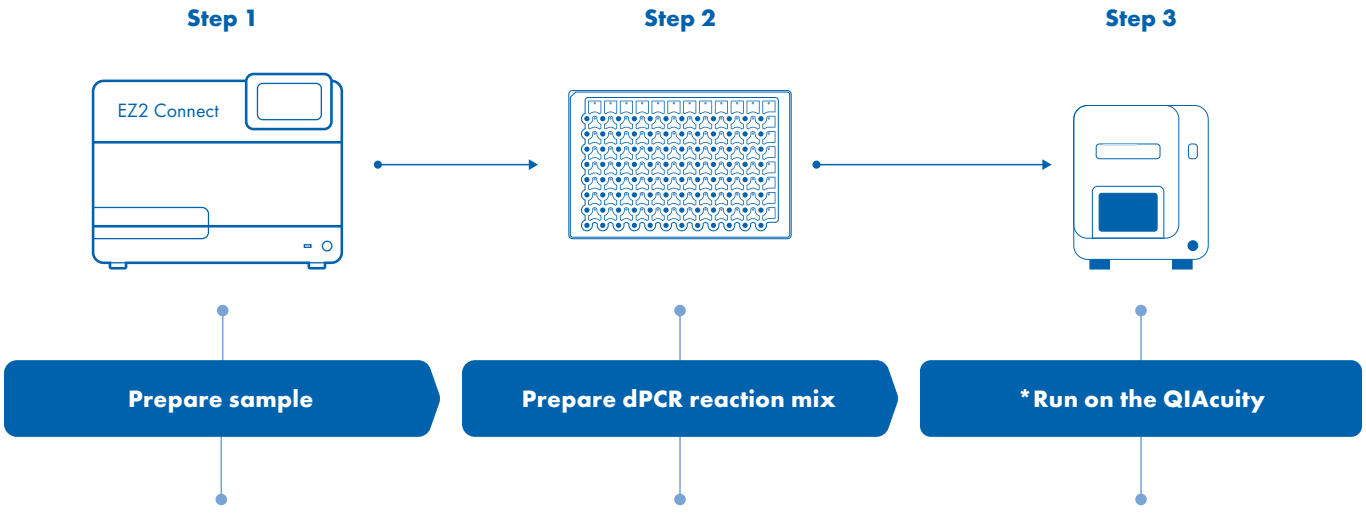

Figure 1. Fast and easy Mycoplasma detection workflow using dPCR allowing same-day results.

RNA and DNA Isolation with the EZ2 Connect protocol for Mycoplasma

The QIAGEN EZ2 Connect represents a new generation of automated sample preparation, designed to streamline the process of purifying DNA and RNA. It allows for the purification of DNA and RNA from up to 24 samples simultaneously, significantly boosting both reproducibility and convenience. This high-throughput capability supports consistent and scalable mycoplasma testing in regulated biomanufacturing environments.

One of the standout features of these EZ2 Connect kits is the use of prefilled reagent cartridges, which simplify the handling process and help ensure consistent results and process safety. The EZ2 Connect protocol for Mycoplasma is adapted from the EZ1&2 Virus Mini Kit v2.0, making it easier and faster to use. Eluates can be obtained in approximately 45 minutes, making the process not only efficient but also time-saving. The simplified protocol minimizes hands-on time and helps reduce operator-dependent variability, which is especially important in GMP-compliant workflows (Figure 3).

The kit is also highly versatile, with a protocol accommodating 400 µL of starting material. The automated protocol is based on magnetic beads, enhancing the efficiency and reliability of the purification process. The fully integrated cartridge design means that each cartridge is used for one sample, reducing the risk of cross-contamination and ensuring the integrity of the results. This closed system format increases biosafety and traceability, both critical for quality assurance in advanced therapy production. Fully automated nucleic acid isolation using EZ2 Connect allows a higher degree of automation in mycoplasma detection workflows. It seamlessly integrates into the QIAcuity RT-dPCR workflow, ensuring upstream consistency and downstream sensitivity.

In summary, the EZ1&2 Virus Mini Kit v2.0 offers a convenient and efficient solution for the simultaneous purification of DNA and RNA, with prefilled, sealed

Figure 3. EZ2 Connect system for automated nucleic acid extraction.

reagent cartridges that make the process straightforward and reliable. Its adaptability, closed cartridge format and automated platform make it an ideal solution for high-confidence pathogen detection in complex sample matrices.

Limit of detection

The detection limit is the positive threshold which is representing the lowest concentration of target nucleic acid that yields a positive signal in 95% of replicate reactions. This parameter is critical for determining the assay's sensitivity, particularly in contamination scenarios. To determine the limit of detection (LOD), Mollicutes preparations were diluted to create suspensions with concentrations of 160, 80, 40, 20, 10, 5 and 0 CFU/mL in DMEM medium containing 10% (v/v) FBS. WHO International Standard for Mycoplasma DNA at dilution levels 640, 160, 40, 10, 2.5, 0.625 and 0 IU/mL were prepared in DMEM medium containing 10% (v/v) FBS. Four individual dilution series were prepared on four different days, and each dilution was analyzed in six replicates, resulting in 24 data points per concentration. This multi-day, replicate-rich design ensures robust statistical confidence. All samples of tested mycoplasma species containing 10 CFU/mL, and the sample containing ▷

the WHO International Standard at 10 IU/mL, tested positive for mycoplasma. This confirms that the assay reliably detects down to 10 CFU/mL or 10 IU/mL with ≥95% hit rate (Table 1 and 2), fulfilling Pharmacopeia expectations for NAT-based methods.

Table 1. Limit of detection for different Mollicutes species

Species/sample	Sensitivity
Acholeplasma laidlawii	5 CFU/mL
Mycoplasma arginini	5 CFU/mL
Mycoplasma fermentans	5 CFU/mL
Mycoplasma gallisepticum	5 CFU/mL
Mycoplasma hyorhinis	5 CFU/mL
Mycoplasma orale	5 CFU/mL
Mycoplasma pneumoniae	5 CFU/mL
Mycoplasma salivarium	10 CFU/mL
Mycoplasma synoviae	10 CFU/mL
Spiroplasma citri	5 CFU/mL
WHO International Standard	10 IU/mL

Table 2. Reproducibility of *Mycoplasma fermentas* detection across different dilutions

	20 CFU/mL				10 CFU/mL				5 CFU/mL			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Hit rate	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Mean value (copies/µL)	55.43				26.42				14.74			
SD	23.94				12.24				8.14			
Out of 24*	24				24				24			
IC mean value (copies/µL)	43.73				43.08				42.41			
IC SD	4.52				5.06				4.91			
IC CV	10.33%				11.74%				11.57%			

**24 replicate reactions were performed per concentration (6 per day across 4 days)*

QIAcuity Mycoplasma Quant Kit workflow detects low contaminations down to 5 CFU/mL

Mycoplasma orale samples were processed according to the QIAcuity Mycoplasma Quant Kit workflow (QIAGEN) or according to Competitor B's workflow. Mycoplasma orale in either DMEM + 10% FCS (DMEM) or 5 x 10^6 CHO cells/mL (CHO Background) was used as a sample in two different concentrations, 10 CFU/mL and 5 CFU/mL. Each concentration and sample matrix was tested in technical replicates, and the mean values are presented below. Mycoplasma was successfully detected in all samples by the QIAcuity Mycoplasma Quant Kit, independent of the sample matrix. This demonstrates the assay's consistent performance even in complex backgrounds such as CHO cells. On the other hand, samples processed according to Competitor B's workflow are considered mycoplasma negative as the measured concentrations are below the threshold of 0.1 copies/µL, which Competitor B uses to evaluate the test results.

As a result, Competitor B's method failed to detect contamination at both 10 CFU/mL and 5 CFU/mL in multiple matrices. These data demonstrate that the QIAcuity Mycoplasma Quant Kit achieves a detection sensitivity of at least 5 CFU/mL, meeting the Pharmacopeia requirements for replacing both the traditional indicator cell culture method and the conventional broth/agar culture method. In contrast, Competitor B's workflow lacks the sensitivity to serve as a complete substitute for regulatory-compliant culture-based detection (Figure 4).

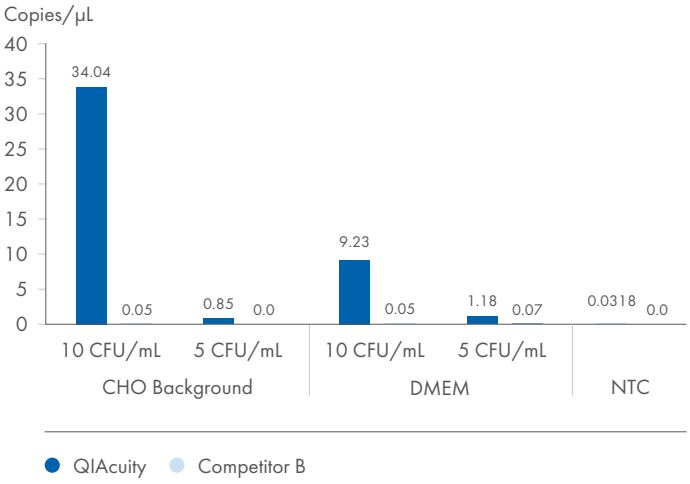

Figure 4. QIAcuity Mycoplasma Quant Kit enables reliable detection down to 5 CFU/mL across sample matrices.

Summary

Reliable mycoplasma detection is essential for ensuring the safety of cell-derived biopharmaceutical products, particularly in advanced therapy manufacturing. The QIAcuity Mycoplasma Quant Kit, combined with the EZ2 Connect automated sample preparation system, offers a fast, sensitive and pharmacopeia-compliant workflow that supports high-throughput mycoplasma testing with minimal hands-on time.

Unlike traditional culture-based assays or DNA-only methods, this RT-dPCR-based solution targets both rRNA and DNA, enabling the detection of low-level contaminations down to 5 CFU/mL across complex sample matrices such as CHO cell suspensions and media. Internal controls and ready-to-use CFU standards provide confidence in assay performance and compliance with European, US and Japanese Pharmacopeia requirements.

Together, the QIAcuity and EZ2 Connect platforms streamline quality control testing, reduce time-to-results to within one day, and enhance risk management during process development, manufacturing and batch release. This robust digital PCR-based approach supports safer, faster and more reliable delivery of biotherapeutic products to patients.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or the user operator manual. QIAGEN instructions for use and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services (or your local distributor). All figures and underlying data are from the QIAGEN R&D Department.

Explore our QIAcuity Mycoplasma Quant Kit at **www.qiagen.com/mycoplasmakit**