Digital PCR applications for cell and gene therapy: High-quality detection of mycoplasma contamination

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Sensitive and rapid testing for mycoplasma contamination

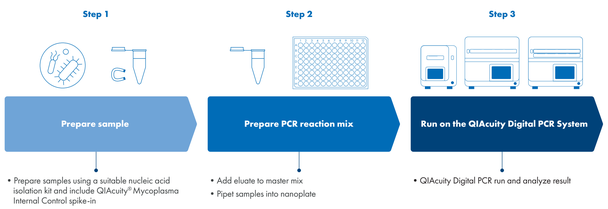
Developing safe and effective biopharmaceuticals requires strict quality controls at all stages of the development process and during manufacturing. The rapid detection of potential impurities is crucial for ensuring high-quality advanced therapy medicinal products (ATMPs). Nucleic acid techniques (NATs) for mycoplasma contamination testing are well established and described by different pharmacopeias worldwide. Some compendia, including the European Pharmacopeia (EP), have announced a revision that will make the validation of NATs more stringent.

Therefore, we present a workflow for detecting mycoplasma presence/absence based on a one-step RT-dPCR (reverse transcription digital PCR) to detect mycoplasma rRNA, as well as DNA. This allows for highly sensitive and robust contamination detection in a variety of sample matrices, such as media containing high salts or high amounts of producer cells. The validation report covering the complete workflow (and adhering to all the necessary criteria established by the guidelines) shows a limit of detection down to 5–10 CFU/mL for the most common mycoplasma species. Based on sequence alignment, at least 127 *Mollicutes* species can be detected.

Furthermore, irreversibly inactivated and experimentally verified standards are crucial to establish and validate robust in-house NAT mycoplasma testing, while avoiding the handling of infectious mycoplasma. This need is met by the Mycoplasma Standard CFU Kits for all 10 compendia-mentioned species. The standard material allows the LOD verification at ≤10 CFU/mL and can also be used as positive control material.

Fast and easy mycoplasma detection workflow using dPCR

Rapid mycoplasma testing that meets the requirements described by the US (USP), Japanese (JP) and European (EP) Pharmacopeia – including the recently announced revisions. Various sample matrices from in-process controls, cell cultures and QC samples can be tested by the validated NAT workflow.



QIAGEN's mycoplasma detection workflow in three steps. Step 1: Nucleic acid extraction with spike-in of Internal Control during the lysis step. Step 2: Setup of dPCR reaction with up to 22.6 µL sample and transfer to a 26k Nanoplate. Step 3: One-step reverse transcription dPCR run on the QIAcuity instrument and subsequent data analysis using the QIAcuity Software Suite.

Significantly enhanced sensitivity using RT-dPCR compared with dPCR mycoplasma testing

In general, mycoplasma cells harbor multiple RNA copies, but only one or a few genomic DNA copies of the analyzed target. Consequently, targeting reversely transcribed RNA in addition to genomic DNA can enhance the sensitivity of the dPCR-based detection method. Differences in RNA expression levels and cluster formation prevent a precise quantification of DNA in the reaction, which is irrelevant for mycoplasma testing that aims for a qualitative result.

Comparison of RT-dPCR and dPCR-based mycoplasma testing

M. fermentans diluted in DMEM + 10% FCS	RT-dPCR				dPCR			
Conc. sample (CFU/mL)	Conc. (copies/µL)	Mean conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)	Conc. (copies/µL)	Mean conc. (copies/µL)	SD (n=3) (copies/µL)	CV (n=3)
10,000	Oversaturated Oversaturated Oversaturated	Oversaturated	NA	NA	98.53 90.73 83.56	90.9	6.11	6.7%
1,000	2878.3 2702.9 2900.7	2827.3	88.44	3.1%	9.32 8.479 9.094	9.0	0.36	4.0%
100	165.1 244.8 289.4	233.1	51.42	22.1%	0.482 0.662 0.822	0.7	0.14	21.2%
10	21.37 53.52 21.48	32.1	15.13	47.1%	0.000 0.000 0.16	0.1	0.08	141.4%
1	0.000 3.714 0.159	1.3	1.71	132.8%	0.000 0.000 0.000	0.0	0.00	NA
R^2 value for the linear regression (from 1000 to 1 CFU/mL)		0.9997				0.9994		

A 10-fold dilution series of *Mycoplasma fermentas* was processed in triplicates according to the QIAcuity Mycoplasma Quant Kit workflow. In addition to the RT-dPCR (dark blue), a dPCR (without the RT step, light blue) was run on the QIAcuity dPCR System. The 10 CFU/mL dilution is highlighted in green (sensitivity criteria met) and red (sensitivity criteria not met) representing RT-dPCR and dPCR results, respectively. Mean concentration (Mean conc.), standard deviation (SD), coefficient of variation (CV) and linearity (R^2) were calculated. Different RNA expression levels and cluster formation of mycoplasma likely are the reason for the variability between the replicates. CFU: Colony forming units; FCS: Fetal calf serum; NA: Not applicable.

The QIAcuity Mycoplasma Quant Kit workflow is validated to meet pharmacopeia requirements and is compatible with a variety of sample matrices

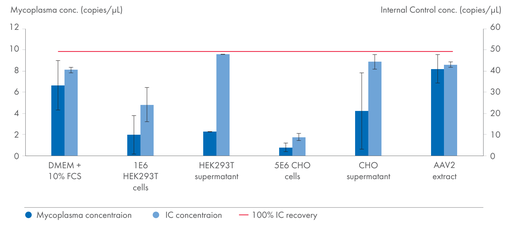
The QIAcuity Mycoplasma Quant Kit workflow has been validated to determine the limit of blank (LoB), limit of detection (LoD), precision, dynamic range, linearity, robustness, and specificity according to the requirements of the most recent guidelines. Using the recommended QIAcuity Mycoplasma Quant Kit workflow can save time and reduce the validation effort.

Limit of detection for different *Mollicutes* species

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

LoD results were determined as part of the QIAcuity Mycoplasma Quant Kit validation report and are shown for *Mollicutes* species mentioned by the EP, USP and JP, as well as the WHO International Standard. IU: International units.

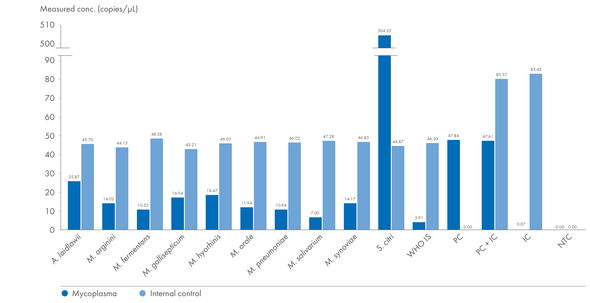
Sample matrix compatibility with the QIAcuity Mycoplasma Quant Kit workflow



Resistance to inhibition in different sample matrices. HEK293T cells and CHO cells were cultivated in DMEM +10% FCS. *Mycoplasma orale* was spiked into the samples at a final concentration of 10 CFU/mL. Samples were processed according to the QIAcuity Mycoplasma Quant Kit workflow. All samples were identified as mycoplasma positive, even though high background cell amounts can lead to PCR inhibition due to saturation of the nucleic acid extraction system.

Validation of dPCR-based mycoplasma testing using mycoplasma standards

Irreversibly inactivated, lyophilized Mycoplasma Standard CFU kits covering the most frequently occurring mycoplasma contaminants enable safe, in-house validation of NAT mycoplasma testing. Mycoplasma standards can replace time-consuming classical cell-culture methods without handling infectious bacteria.



Validation of QIAGEN's mycoplasma detection workflow without handling infectious bacteria. Irreversibly inactivated QIAcuity Mycoplasma Standards were dissolved in DMEM + 10% FCS (final concentration 10 CFU/mL). WHO International Standard (IS) was dissolved in nuclease-free water and diluted to 10 IU/mL in DMEM + 10% FCS. Samples were processed according to the QIAcuity Mycoplasma Quant Kit workflow. All tested standards show a clear, positive mycoplasma signal (dark blue, LOB_{qPCR} = 0.144). Mycoplasma Internal Control (light blue) was spiked-in as a process control and recovered constantly at 74% on average. IC: Internal control; NTC: No-template control; PC: Positive control.

Summary

Detecting the presence or absence of mycoplasma using the QIAcuity Mycoplasma Quant Kit workflow offers several advantages:

- A dPCR-based total nucleic acid workflow allows increased sensitivity that meets Pharmacopeia standards.
- Reduced turnaround time allows a faster response in case of contamination, which is highly suited for ATMPs with a short shelf-life.
- A comprehensive validation report assessing the performance of the workflow can reduce validation efforts.
- Validation of the complete workflow without handling infectious mycoplasma possible with 10 different QIAcuity Mycoplasma Standard CFU Kits.

For more information about the QIAcuity Mycoplasma Quant Kit and Standards:

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or 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).

Sample to Insight