CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Analytics Analytical development & QC

Safety assays for quality control of ATMPs: addressing mycoplasma detection challenges

Jens Björkman and Andreas Hecker

In biotherapeutic manufacturing, mycoplasma testing plays an important role in ensuring product safety, but current methods do not always align with the speed required in modern processes. Culture-based assays, although accepted, can take several weeks to generate results. This article looks at how reverse transcription digital PCR (RT-dPCR) can be used as an alternative. The approach enables detection down to 5–10 CFU/mL and performs reliably even in complex sample matrices. When combined with automated nucleic acid extraction, it allows testing to be completed within a single working day.

Cell & Gene Therapy Insights 2026; 12(3), 273–283 · DOI: 10.18609/cgti.2026.035

THE REGULATORY CHALLENGE OF MYCOPLASMA TESTING IN ATMP MANUFACTURING

Mycoplasma detection remains a critical component of safety testing in the manufacturing of advanced therapy medicinal products (ATMPs). As these products move toward clinical use, ensuring safety and consistency is essential. ATMP manufacturing involves extensive cell handling, genetic modification and large-scale expansion steps, creating multiple opportunities for microbial contamination.

Mycoplasma, a common contaminant in mammalian cell cultures, poses a particular risk due to its ability to proliferate undetected and affect cell behavior, phenotype and productivity. Throughout manufacturing processes, fast and reliable mycoplasma detection is essential for enabling timely decision-making and ensuring product quality before clinical use.

Pharmacopeial frameworks in Europe, the US, and Japan specify core safety requirements for ATMPs, including tests for sterility, bacterial endotoxins and mycoplasma. While traditional culture-based assays remain pharmacopeia-accepted, they require extended incubation periods, often up to 28 days, and involve handling viable organisms. These characteristics make them poorly suited to the rapid manufacturing timelines and short shelf

lives associated with many cell-based therapies.

Pharmacopeias also define criteria for replacing culture-based methods with nucleic acid amplification techniques (NATs), provided proper validation is conducted. The required sensitivity for NAT-based methods is the detection of:

- 10 CFU/mL to replace the broth/agar culture method
- 100 CFU/mL to replace the indicator cell culture method

For autologous therapies, where the manufacturing window between patient cell collection and reinfusion is narrow, rapid safety testing is particularly important. These pressures have led manufacturers to increasingly evaluate molecular approaches that can be integrated into modern ATMP workflows.

ANALYTICAL REQUIREMENTS FOR NAT-BASED MYCOPLASMA DETECTION

To replace traditional culture methods, NAT-based assays must demonstrate:

- High analytical sensitivity at or below 10 CFU/mL
- Detection in ≥95% of replicate reactions at the defined limit of detection (LOD)
- Coverage across relevant mollicute species
- Robust performance in complex sample matrices
- Reproducibility suitable for regulated environments

In addition to analytical sensitivity, time-to-result and workflow robustness are critical considerations in ATMP manufacturing settings.

RT-dPCR AS A HIGH-SENSITIVITY MOLECULAR APPROACH

Molecular methods, including quantitative PCR (qPCR) and digital PCR (dPCR), eliminate the need for culturing organisms, significantly reducing time to results. Among these, dPCR has attracted increasing interest due to its high precision, reproducibility, and independence from standard curves.

dPCR enables absolute quantification by partitioning samples into a large number of individual reaction wells prior to amplification. This partitioning reduces susceptibility to inhibitors commonly found in complex sample matrices and improves reproducibility by eliminating reliance on standard curves.

Studies have demonstrated the superior reproducibility of dPCR compared with qPCR [1,2]. One study showed reduced variability between operators when quantifying plasmid DNA by dPCR compared with qPCR [1]. Another demonstrated high inter-laboratory reproducibility when detecting a rare genomic variant at fractional abundances below 0.2%, a level of sensitivity not achievable with qPCR[2]. These findings illustrate the precision attainable with dPCR in regulated environments.

Targeting both DNA and rRNA using reverse transcription dPCR (RT-dPCR) has been shown to improve sensitivity, particularly at low levels of mycoplasma contamination [3]. Viable mycoplasma cells contain multiple rRNA copies, and incorporating reverse transcription enhances detection sensitivity at low contamination levels (Table 1).

ANALYTICAL VALIDATION USING THE QIAcuity Mycoplasma Quant Kit

The QIAcuity Mycoplasma Quant Kit is an RT-dPCR kit for quantifying mycoplasma

TABLE 1

Comparison of reverse transcription digital PCR and digital PCR for mycoplasma quantification across a dilution series.

M. fermentans diluted in DMEM + 10% FCS conc. (CFU/mL)	RT-dPCR				dPCR			
	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	NA NA NA	NA	NA	NA	98.53 90.73 83.56	90.9	6.11	6.7
1000	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 1000–1 CFU/mL)		**0.9997**				**0.9994**		

Mycoplasma fermentans diluted in DMEM + 10% FCS was analyzed using RT-dPCR and dPCR. RT-dPCR showed increased sensitivity at lower concentrations (≤10 CFU/mL) compared with dPCR. Linear regression demonstrated robust quantitative performance (R^2 = 0.9997 for RT-dPCR and 0.9994 for dPCR; n = 3). Conc: Concentration; DMEM: Dulbecco's Modified Eagle Medium; dPCR: Digital PCR; FSC: Fetal bovine serum; RT-dPCR: reverse transcription dPCR.

by isolating and detecting total nucleic acid (rRNA and DNA). The kit includes an internal control to monitor extraction efficiency and PCR inhibition.

Performance of the workflow was evaluated using the QIAcuity Mycoplasma Quant Kit, with additional assessment conducted at CCRM Nordic to reflect application in relevant ATMP sample types.

To characterize assay performance at low contamination levels, the LOD was assessed using the QIAcuity Mycoplasma Quant Kit in a replicate-rich study design consistent with pharmacopeial expectations. In this context, LOD is defined as the lowest concentration of target nucleic acid that yields a positive result in at least 95% of replicate reactions.

Mollicutes preparations were diluted to concentrations of 160, 80, 40, 20, 10, 5 and 0 CFU/mL in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Four independent dilution series were prepared on four different days, and each concentration was analyzed in six replicates, resulting in 24 data points per concentration.

Using this approach, detection down to 5 CFU/mL was consistently achieved, exceeding the pharmacopeial sensitivity threshold of 10 CFU/mL for replacement of broth/agar culture methods (Table 2 and 3).

INDEPENDENT PERFORMANCE EVALUATION BY CCRM NORDIC

A dilution series evaluated at CCRM Nordic confirmed the kit's ability to detect Mycoplasma at the LOD of 10 CFU/mL as

TABLE 2

Limit of detection across multiple *Mollicutes* species using reverse transcription digital PCR.

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
Siroplasma citri	5 CFU/mL
WHO International Standard	10 IU/mL

Sensitivity of the QIAcuity Mycoplasma Quant Kit was evaluated across multiple mollicutes species. Detection was achieved at or below 10 CFU/mL for all species tested, meeting pharmacopeial requirements for NAT-based mycoplasma detection.

TABLE 3

Detection of *Mycoplasma arginini* dilution series evaluated at CCRM Nordic.

	Mycoplasma (FAM)					Internal control (HEX)			
	Conc. (cp/µL; dPCR rxn)	CI(95%; dPCR rxn)	CI(95% cp/µL; dPCR rxn)	Expected	(CFU/mL)	Conc. (cp/µL; dPCR rxn)	CI(95%; dPCR rxn)	Expected (cp/µL)	IC recovery (%)
Qiagen STD 10 CFU/mL *M. arginini* 1	11.934	0.130	1.549	POS	10.0	88.754	0.048	105.9	84
Qiagen STD 10 CFU/mL *M. arginini* 2	11.810	0.132	1.557	POS	10.0	82.897	0.050	105.9	78
Qiagen STD 5 CFU/mL *M. arginini*	4.996	0.203	1.015	POS	5.0	76.337	0.053	105.9	72
Qiagen STD 2.5 CFU/mL *M. arginini*	2.464	0.289	0.712	POS	2.5	77.148	0.052	105.9	73
ENTC	0.056	1.475	0.082	Neg	0.0	80.820	0.052	105.9	76
POS ctrl	55.163	0.061	3.392	50 cp/µL	0.0	0.000	–	0	–

A dilution series of *M. arginini* was analyzed to assess detection performance and internal control recovery across concentrations. Results confirm reliable detection at low CFU levels under CCRM Nordic testing conditions. 1 mL Media (50–50 mix to CryoStor® CS10 and CliniMACS® PBS/EDTA Buffer) was used to dissolve Qiagen *M. arginini* standard.
Conc.: Concentration.

well as an efficient IC recovery. Additionally, the linear regression with $R^2 > 0.995$, confirming robust quantitative performance across the tested range (Table 3 and Figure 1).

PERFORMANCE IN COMPLEX SAMPLE MATRICES

Following analytical validation of assay sensitivity, performance was further assessed in complex sample matrices relevant to ATMP manufacturing. Serial dilutions of Mycoplasma orale ranging from 160 CFU/mL to 5 CFU/mL were prepared in DMEM supplemented with 10% fetal calf serum (FCS).

All 24 replicates yielded positive results at 5 CFU/mL, demonstrating reliable detection in serum-containing media. Scatter plot analysis showed a consistent decrease in signal intensity with decreasing mycoplasma concentration, supporting quantitative reliability (Figure 2).

Detection down to 5 CFU/mL was consistently achieved across complex sample matrices, positioning RT-dPCR as a sensitive alternative capable of meeting current pharmacopeial requirements.

INTEGRATION INTO A PHARMACOPEIA-COMPLIANT WORKFLOW USING EZ2 CONNECT & QIACUITY

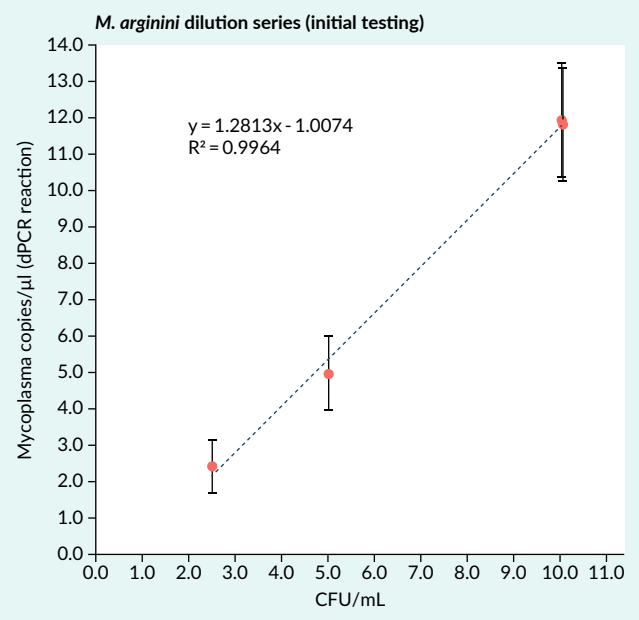
Pharmacopeial standards allow the use of NATs as an alternative to conventional mycoplasma culture assays when appropriately validated. In a regulated ATMP environment, upstream nucleic acid isolation is a critical determinant of overall assay performance.

The EZ2 Connect platform enables automated purification of both RNA and DNA using prefilled, sealed reagent cartridges. The system supports parallel processing of up to 24 samples, reduces hands-on time and enhances biosafety and traceability.

Using this approach, eluates can be obtained in approximately 45 minutes.

FIGURE 1

Linearity assessment of reverse transcription digital PCR-based mycoplasma quantification.



Serial dilutions demonstrated robust quantitative performance with $R^2 > 0.995$ across the tested concentration range.

Extracted nucleic acids are combined with the QIAcuity Mycoplasma Quant Master Mix and dispensed into a 26k Nanoplate, followed by RT-dPCR analysis on the QIAcuity Digital PCR system.

The complete workflow, from extraction on EZ2 Connect to data analysis on QIAcuity, can be completed within 6–7 hours, enabling same-day mycoplasma detection and supporting faster decision-making in a QC setting (Figure 3).

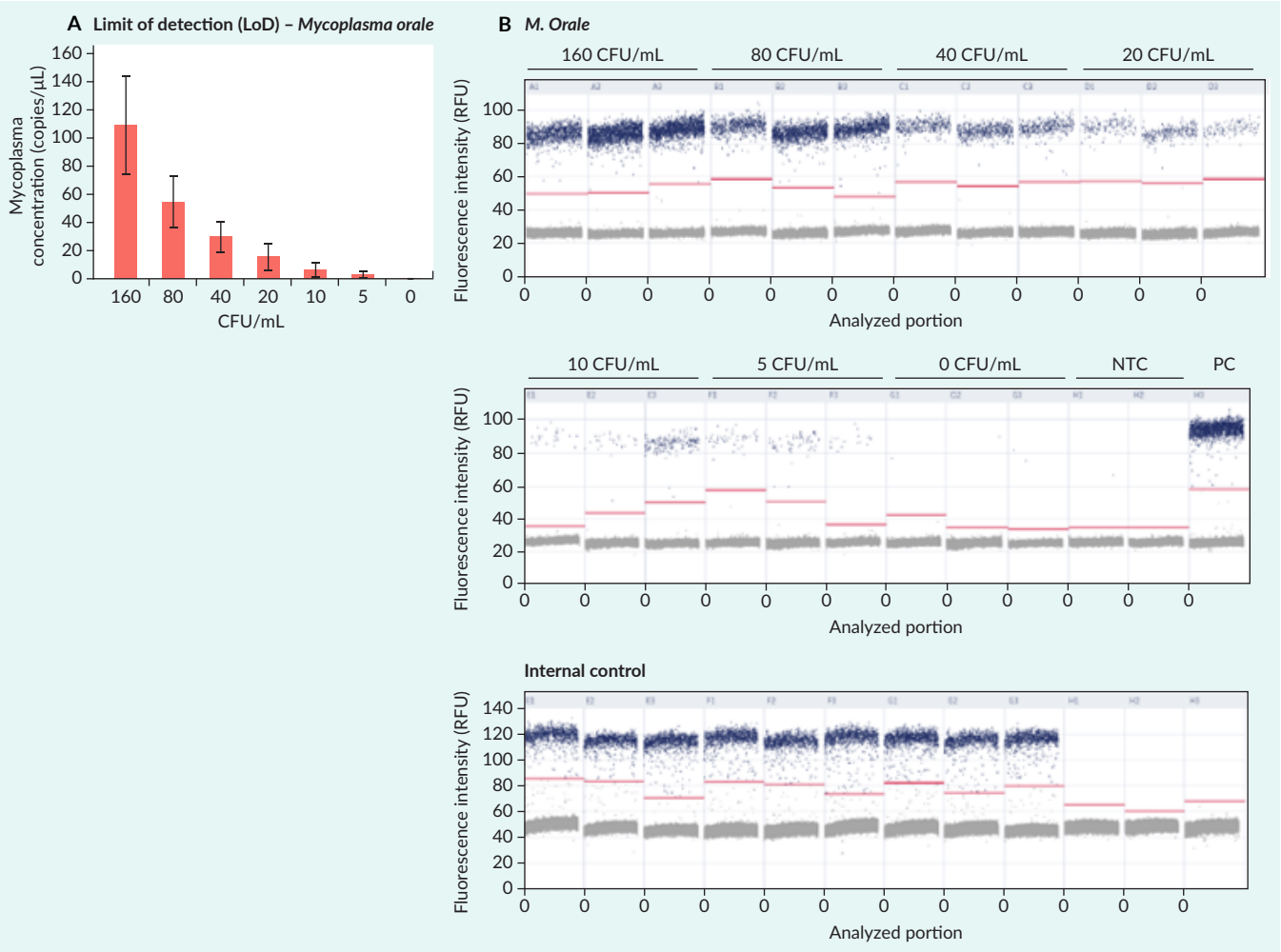
Importantly, integration of automated extraction upstream of RT-dPCR does not compromise analytical sensitivity and supports robust detection even in complex matrices such as serum-containing media and cell suspensions.

INDEPENDENT IMPLEMENTATION PERSPECTIVE IN ATMP MANUFACTURING

From an ATMP manufacturing perspective, reproducibility and robustness are essential.

FIGURE 2

Limit of detection assessment for *Mycoplasma orale* using reverse transcription digital PCR.



(A) Quantitative detection across serial dilutions from 160 to 0 CFU/mL in DMEM supplemented with 10% FBS. **(B)** Representative 1D scatter plots demonstrating partition separation across concentrations, including reliable detection at 5 CFU/mL. Detection met the ≥95% positivity criterion consistent with pharmacopeial expectations for NAT-based method replacement.

Experience in regulated environments has demonstrated that dPCR offers reduced operator-dependent variability and strong inter-laboratory reproducibility.

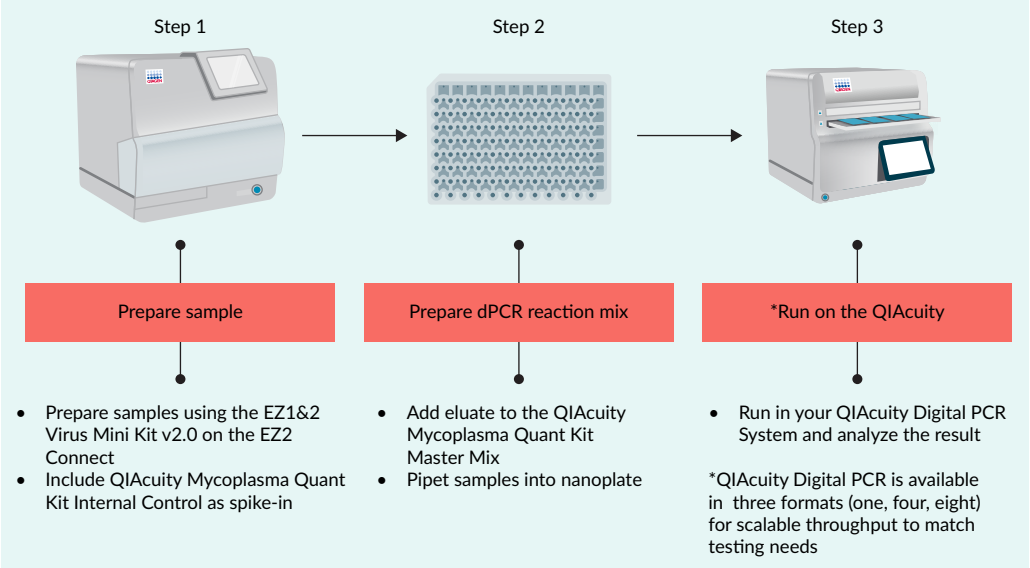
The combination of automated nucleic acid isolation using EZ2 Connect and absolute quantification using QIAcuity supports consistent performance across operators and sites. This level of reproducibility is particularly valuable in GMP-compliant ATMP manufacturing, where traceability and consistency are critical.

SUMMARY

Ensuring the safety of ATMPs requires sensitive, reproducible and time-efficient mycoplasma detection strategies that can be integrated into modern manufacturing workflows. While traditional culture-based methods remain pharmacopeia-accepted, their long incubation times and reliance on viable organisms limit their suitability for products with short shelf lives.

FIGURE 3

Pharmacopeia-compliant NAT workflow integrating automated nucleic acid extraction on EZ2 Connect with reverse transcription digital PCR analysis on the QIAcuity Digital PCR system.



The sample-to-result process can be completed within a single working day.

RT-dPCR-based workflows using the QIAcuity Mycoplasma Quant Kit demonstrate detection down to 5–10 CFU/mL, reaching or exceeding the 10 CFU/mL sensitivity required for replacement of broth/agar culture methods. Linear assay performance, replicate-rich validation and robust detection in complex matrices support the suitability of this approach for regulated environments.

When combined with automated RNA and DNA isolation using EZ2 Connect and analysis on the QIAcuity Digital PCR system, the workflow enables same-day results within a pharmacopeia-compliant NAT framework.

Together, RT-dPCR and automated sample preparation provide a sensitive and reproducible solution for mycoplasma testing in ATMP manufacturing.

Q&A

Jens Björkman (left), **Andreas Hecker** (right)

Q Since assay sensitivity is dependent on RNA and DNA, are live mycoplasma needed during the development and validation of LOD?

JB It is necessary to work with mycoplasma cells, however, bringing in live mycoplasma cells for validation can also be a problem in a lab environment where mycoplasma should be kept away from cells. Using inactivated mycoplasma standards is quite common.

Q What was the range of the GC and CFU ratio for the mycoplasma-used-challenge sensitivity?

JB For the small tests, we used M. arginini, and that had a GC:CFU ratio of ~2.1. This ratio can vary by mycoplasma species.

Q How quickly can the Mycoplasma Quant Kit analysis be performed?

JB The mycoplasma quantification kit analysis can be performed within 6–7 hours. This involves performing the sample extraction, setting up, and running the dPCR. Any steps involving liquid handling are now automated in the workflow, further shortening the time to perform the analysis.

Q Is safety testing limited to mycoplasma, sterility, and endotoxin detection?

JB These are the core attributes that are commonly tested, however, depending on the specific product, it may be necessary to perform other types of safety tests. For example, it may be necessary to test for viruses, whole-cell DNA, and proteins. When working with viral vector-based therapies, it will be necessary to assess vector copy numbers and replication-competent virus components – replication-competent lentivirus or replication-competent retrovirus.

AH There are stringent regulatory requirements detailed in the guidelines of different pharmacopeia. Depending on the modalities being worked on, it is important to refer to the corresponding chapters within the guidelines as they give a detailed description of required tests.

Q When will the new validation report for the EZ2 protocol become available?

AH The validation of the updated EZ2 protocol has been completed. The replicate-rich study design included 1,024 replicates per strain to ensure robust statistical confidence. The finalized validation report is now available.

REFERENCES

1. Isham IM, Najimudeen SM, Cork SC, Gupta A, Abdul-Careem MF. Comparison of quantitative PCR and digital PCR assays for quantitative detection of infectious bronchitis virus (IBV) genome. *J. Virol. Methods* 2024; 324, 114859.
2. Whale AS, Devonshire AS, Karlin-Neumann G, *et al*. International interlaboratory digital PCR study demonstrating high reproducibility for the measurement of a rare sequence variant. *Anal. Chem.* 2017; 89(3), 1724–1733.
3. QIAGEN. Fast and sensitive mycoplasma detection workflow using RT-dPCR with same-day results. https://www.qiagen.com/pn/resources/download.aspx?id=bb759a31-866b-4dee-afdc-2bb26f95aaa5&lang=en

BIOGRAPHIES

Jens Björkman has over 15 years of experience developing/applying nucleic acid analysis techniques, including assay design/validation, isolation, quality control, qPCR, dPCR, NGS, and associated automation. Previously, he was a Director and COO of regulatory compliant CRO (ISO17025/GLP) serving academic/industry/healthcare clients.

Jens Björkman, Analytical Development Manager, CCRM Nordic

Andreas Hecker has over 8 years of experiences in managing product portfolios in various application areas including BioPharma QC solutions for the QIAcuity, QIAGEN's digital PCR platform. Previous roles were related to technical product service and product training at a different life science tools provider.

Andreas Hecker, Associate Global Product Manager, dPCR Consumables, QIAGEN

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The authors have no conflicts of interest.

Funding declaration: The authors received no financial support for the research, authorship and/or publication of this article.

AI process statement: This article is based on a webinar developed and presented by the authors. The webinar was transcribed into text, and AI tools (ChatGPT) supported non-creative tasks including the organization of the source material by human editors, removing repetition and non-substantive dialogue from raw transcripts and correcting spelling and grammar. All content ultimately reflects human expertise, ethical rigor, and scientific integrity. Following Editor review, the final article was reviewed, refined, edited and approved by the authors.

BioInsights encourages transparent, responsible, and verifiable use of AI as a supporting tool; never a creative substitute. All content ultimately reflects human expertise, ethical rigor, and scientific integrity. See our full AI policy statement for more information.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article was developed by BioInsights' Editorial team of subject matter experts using expert insights shared during the webinar 'Safety assays for quality control of ATMPs: improved mycoplasma detection for cell-based biotherapeutics', alongside supporting presentation materials to create a refined, editorial-led analysis.

Webinar conducted: Apr 30, 2025.

Revised manuscript received: Apr 7, 2026.

Publication date: Apr 29, 2026.

Bring your life-saving therapies to market faster, with confidence

Explore our knowledge hub to see how we can advance your cell and gene therapy development.

Here's what you'll find:

- Scalable digital PCR solutions and standardized workflows for:
  - Absolute quantification of AAV genome titers
  - Robust detection of residual host cell DNA contaminants
  - Confirmation of CRISPR-mediated editing events in CAR-T cells
- Grants and partnerships to help grow your business
- Bespoke packages and services to address your company's needs

Visit **www.qiagen.com/pharma-biotech** or scan the QR code to learn more.

Sample to Insight