iNtRON Biotechnology

Mycoplasma testing of Biopharmaceuticals & ATMP

Technical Report

| e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)

High-precision detection of mycoplasma DNA using the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)

Abstract

Mycoplasma contamination represents a critical risk in the production of biopharmaceuticals and cell-based medicinal products, including advanced therapy medicinal products (ATMPs). Even in the absence of visible signs, mycoplasmas can adversely affect cell culture performance, product yield, quality, and safety. Regulatory agencies worldwide require manufacturers to demonstrate the absence of mycoplasmas in all production batches prior to product release.

The **e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)** provides a rapid, reliable, and guideline-compliant method to detect mycoplasma contamination in cell-based manufacturing processes. The assay offers comprehensive coverage of international pharmacopeia standards (EP, USP, JP, and Korea MFDS), enabling simultaneous detection of compendial reference species (including *A. laidlawii, M. arginini, M. gallisepticum, M. fermentans, M. hyorhinis, M. orale, M. pneumoniae, M. salivarium, M. synoviae,* and *S. citri*) within a single integrated panel. Panelized primers and probes, developed through extensive sequence alignment and in-silico validation, ensure high specificity and meet established detection limits without cross-reactivity with non-target microorganisms. Additionally, internal and external controls ensure precise and reproducible measurements across standard sample types, supporting assay reliability. Testing can be completed within one hour, offering a significant time advantage over traditional mycoplasma culture methods.

This technical report provides a guideline-compliant, user-friendly solution for routine mycoplasma testing and facilitates integration into GMP-compliant quality control workflows, supporting regulatory compliance and protecting product integrity.

Introduction

Mycoplasmas have emerged as critical contaminants in cell culture systems, in addition to their known clinical relevance. Since they are naturally present in plant and animal tissues, any culture medium containing plant- or animal-derived supplements is susceptible to mycoplasma contamination. Due to their extremely small size (0.1 to 0.8 μm) and lack of a rigid cell wall, mycoplasmas can adopt variable shapes and pass through standard sterile filters, making their way into cell cultures via raw materials or culture media components. The most common sources of contamination are laboratory personnel and pre-contaminated cell lines, which can spread the organism through cross-contamination.

Mycoplasmas are not visible under standard light microscopy and typically do not produce noticeable changes in cell morphology, which often leads to undetected contamination. However, their presence can significantly impact cell growth, metabolism, and the quality of therapeutic proteins produced by host cells.

Mycoplasma contamination is a critical concern in the quality control of cell cultures and biopharmaceuticals, primarily due to the difficulty of detecting low-level infections. Traditional compendial methods namely, the Culture Method and the Indicator Cell Culture Method have long been the regulatory standard due to their high sensitivity and reliability. Although non-compendial assays (e.g., enzyme-based or bioluminescence tests) offer faster turnaround and ease of use, they typically lack the sensitivity required to detect contamination levels ≤10 CFU/mL, as mandated by major pharmacopoeias.

In response to the need for faster and equally sensitive alternatives, nucleic acid amplification techniques (NAT), such as PCR, have been recognized in major pharmacopoeia guidelines including European Pharmacopoeia (EP 2.6.7), United States Pharmacopeia (USP <63>), Japanese Pharmacopoeia (JP G3-14-170), Korea MFDS(2022-58). These guidelines allow the use of NAT under specific conditions, provided the method demonstrates equivalent or superior sensitivity, specificity, and robustness. Validation requirements across EP, USP, and JP commonly include detection of ≤10 CFU/mL of key Mycoplasma species, as well as assessments of LOD, specificity, and performance in routine testing. While each regulatory body varies slightly in its focus, all three recognize the growing importance of NAT in overcoming the time and resource constraints of traditional culture-based methods.

Table 1 : Major Mycoplasma Species Detectable According to Guidelines

Mycoplasma species	EP	USP	JP	MFDS
A. laidlawii	●	●	●	●
M. arginini	●	-	●	●
M. fermentans	●	●	●	●
M. gallisepticum	●[A]	●	-	●[A]
M. hyorhinis	●	●	●	●
M. orale	●	●	●	●
M. pneumoniae	●[A]	●	●	●[A]
M. salivarium	-	-	●	-
M. synoviae	●	●	●[B]	●[B]
S. citri	●	-	●[B]	●[B]

A : (Alternative reference species) In the European Pharmacopoeia (EP) and Korea MFDS, it is available as *Mycoplasma pneumoniae* or *Mycoplasma gallisepticum.*

B : (Insect, Plant, or Avian-derived species) In the Japanese Pharmacopoeia (JP), species of insect or plant origin (e.g., *Spiroplasma citri*) should also be tested in addition to the listed species. When avian cells or materials are used, avian-derived species (e.g., *Mycoplasma synoviae*) may also be considered for inclusion in the test panel.

Mycoplasma Testing Methods

e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)

The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is designed to specifically detect mycoplasma by targeting highly conserved regions of Mycoplasma DNA. The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is suitable for rapid and accurate testing of mycoplasma negative results, providing confirmation within 1 hour (52 minutes). The e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) is an in vitro nucleic acid amplification test optimized for the detection of Mycoplasma in K562 cell culture, according to the Nucleic Acid Amplification Tested (NAT) guidelines for Mycoplasma described in chapter 2.6.7 of the European Pharmacopoeia, with respect to specificity, sensitivity/detection limit, and robustness. The kit was developed according to the CFU specifications provided by suppliers of test materials (e.g., European Directorate for the Quality of Medicines & Health Care and Ministry of Food and Drug Safety).

- Validation for the production of Biopharmaceuticals & Advanced Therapy Medicinal Products(ATMP)
- Rapid contamination detection using Real-time PCR : 53 min
- Prevention of carry-over contamination using Uracil-DNA Glycosylase (UDG)
- Application of IC & EC system to verify the validity of qPCR

Table 2 : Detection Performance of e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO) for Pharmacopoeial Guidelines

Concentration (10 CFU/mL)	Detection Rate (%)
Acholeplasma laidlawii	100 % (8/8) (Positive/Replicates)
Mycoplasma arginini	
Mycoplasma fermentans	
Mycoplasma gallisepticum	
Mycoplasma hyorhinis	
Mycoplasma orale	
Mycoplasma pneumoniae	
Mycoplasma salivarium	
Mycoplasma synoviae	
Spiroplasma citri	

Sample Preparation

Extract nucleic acids from test samples such as cell culture supernatants, cell lysates, or other relevant materials to be used as templates for PCR. The procedure is compatible with conventional DNA extraction methods. For low-throughput applications, manual DNA extraction can be performed using the G-spin™ Total DNA Extraction Mini Kit. For high-throughput workflows, an automated system such as the Miracle-AutoXT Automated Nucleic Acid Extraction System, in combination with the AutoXT Extraction Kit, enables simultaneous processing of multiple samples. Both methods obtain DNA samples in less than 30 minutes, making them ready for PCR quickly.

A. G-spin™ Total DNA Extraction Mini Kit

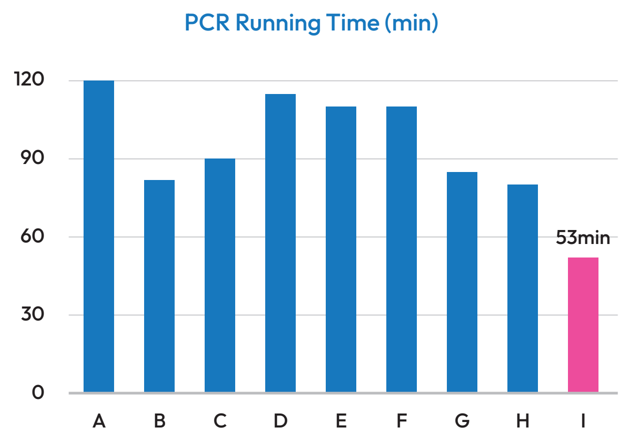
B. Miracle-AutoXT Automated Nucleic Acid Extraction System

A. Manual type : Genomic DNA extraction method using a manual system, We recommend using the G-spin™ Total DNA Extraction Kit (Cat No. 17045, 17046), which can extract genomic DNA from the cell.

B. Automated type : Genomic DNA extraction method using a Automated system, We recommend using the Miracle-AutoXT Automated Nucleic Acid Extraction System (Cat No. IMC-NC15PLUS) and AutoXT Total gDNA Kit (Cat No. 17188-48, 17188-96), which can extract genomic DNA from the cell.

Real-time PCR setup

Prepare the detection mix by dispensing the components into each Real-time PCR tube as described below. After centrifugation, place the tubes into a Real-time PCR system and run the reaction. Once the reaction is complete, interpret the results using the cut-off values provided in the table below. The FAM channel detects the mycoplasma target, and the HEX channel detects the internal positive control (IPC). Manual Threshold Setting : Adjust the threshold line manually on the amplification curve graph. Position the threshold at approximately 5–10% of the fluorescence saturation level observed in the positive control reaction. An IPC Ct value > 27 may result from competition with a high concentration of target DNA. This is considered normal and does not indicate assay failure.



A~H : Other company's NAT-based Real-time PCR kit
I : e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)

Figure 1 : Evaluation of Detection Performance at 10 CFU/mL under Rapid Testing Conditions. The e-Myco™ VALiD-Q Real-Time PCR Kit (v3.0 PRO), which can perform mycoplasma testing in less than 1 hour, is faster than competitive products and has been proven to achieve 100% detection using a certified mycoplasma reference standard panel with a concentration of 10 CFU/mL.

Results

Limit of Detection (LOD) test

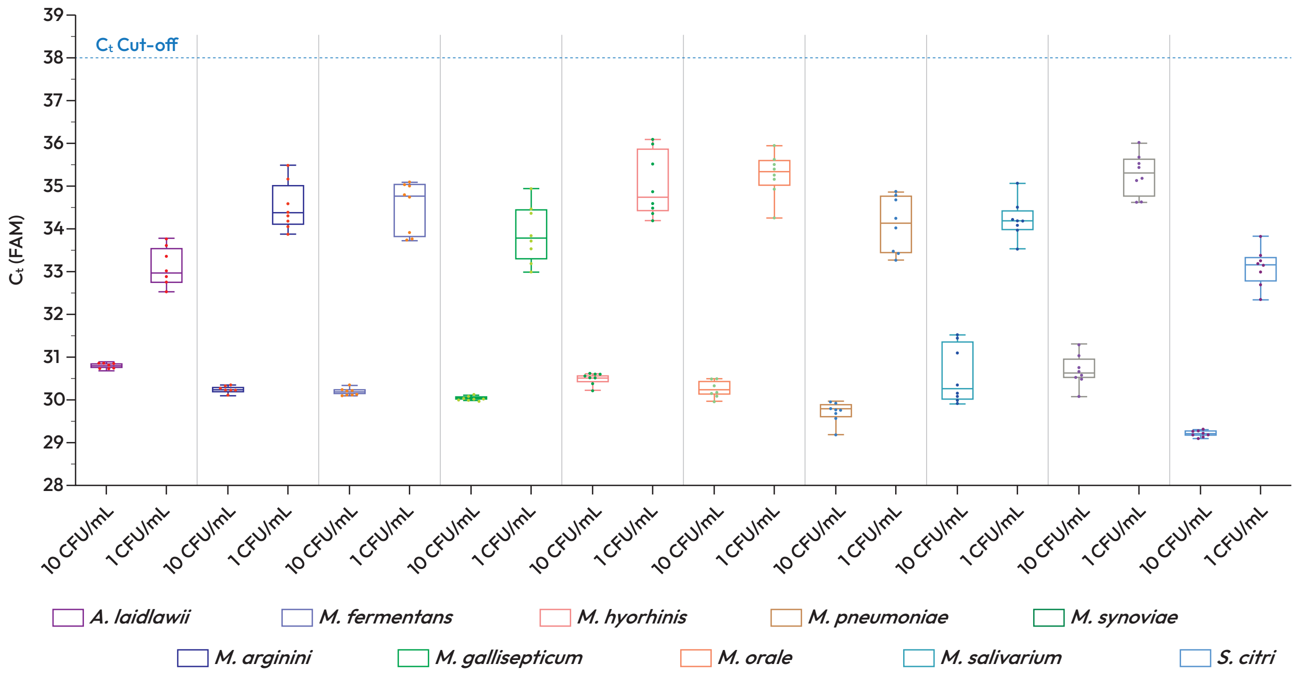


Figure 2 : Detection limit evaluation of the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO). To evaluate the detection sensitivity of the assay, genomic DNA from ten quantified Mycoplasma strains(*A. laidlawii, M. arginini, M. gallisepticum, M. fermentans, M. hyorhinis, M. orale, M. pneumoniae, M. salivarium, M. synoviae*, and *S. citri*) was serially diluted to 10 CFU/mL and 1 CFU/mL. Nucleic acids were extracted directly from culture media and tested using the e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO). All species showed positive detection at the lowest dilution level, confirming a detection limit of 1 CFU/mL for each Mycoplasma species tested.

Table 3 : Evaluation of repeatability and linearity in the Mycoplasma real-time PCR assay.

Mycoplasma Species	Concentration (CFU/mL)	Replicates	Mean Ct (FAM)	*S.D. of Ct (FAM)	**CV(%) of Ct (FAM)	Detection rate(%)
A.laidlawii	10	24	30.95	0.19	0.61	100.0
	1	24	33.28	0.41	1.23	100.0
M. arginine	10	24	30.49	0.25	0.81	100.0
	1	24	34.78	0.61	1.76	100.0
M. fermentans	10	24	30.54	0.33	1.09	100.0
	1	24	34.38	0.64	1.85	100.0
M. hyorhinis	10	24	30.78	0.25	0.82	100.0
	1	24	35.07	0.57	1.63	100.0
M. orale	10	24	30.16	0.29	0.96	100.0
	1	24	35.19	0.48	1.35	100.0
M. pneumoniae	10	24	30.01	0.27	0.90	100.0
	1	24	34.13	0.64	1.88	100.0
M. synoviae	10	24	30.67	0.67	2.18	100.0
	1	24	35.28	0.58	1.65	100.0
M. gallisepticum	10	24	30.29	0.22	0.71	100.0
	1	24	33.90	0.61	1.81	100.0
M.salivarium	10	24	30.33	0.65	2.15	100.0
	1	24	34.36	0.50	1.44	100.0
S.citri	10	24	29.39	0.19	0.66	100.0
	1	24	33.09	0.44	1.34	100.0

*SD : standard deviation
**CV : coefficient of variation

Testing was performed with 24 replicates for each of the ten Mycoplasma species (*A. laidlawii, M. arginini, M. gallisepticum, M. fermentans, M. hyorhinis, M. orale, M. pneumoniae, M. salivarium, M. synoviae*, and *S. citri*) at concentrations of 10 and 1 CFU/mL to verify assay precision and confirm the 95% positive detection rate. All strains exhibited consistent, concentration-dependent Ct values with minimal variability, demonstrating reliable performance at 10 CFU/mL. Consequently, analytical detection limits were validated for all species, and their respective Limit of Detection (LOD) values were established using Probit analysis (Table 3).

Robustness test

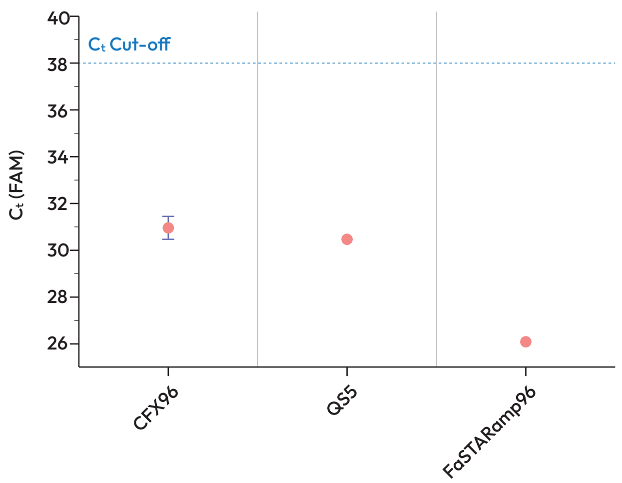


Figure 3 : Reliability and reproducibility of the Mycoplasma Target (FAM) assay under different PCR instrument conditions. Real-time PCR was performed using identical samples (10 CFU/mL *A. laidlawii* DNA) across three instruments (CFX96, QS5, and FaSTARamp96) to evaluate assay robustness..

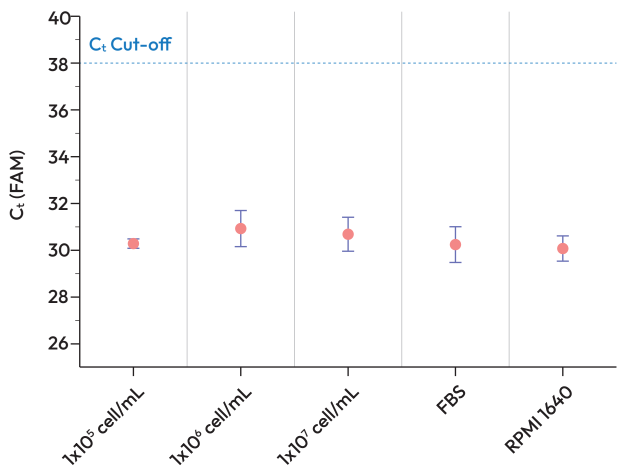


Figure 5 : Reliability and reproducibility of the Mycoplasma Target (FAM) assay under different sample matrix conditions. Identical samples (10 CFU/mL *A. laidlawii* DNA) were evaluated in the presence of cultured cells (1×10^5, 1×10^6, and 1×10^7 cells/mL), FBS, and fresh media (RPMI 1640) to assess robustness in various biological matrices.

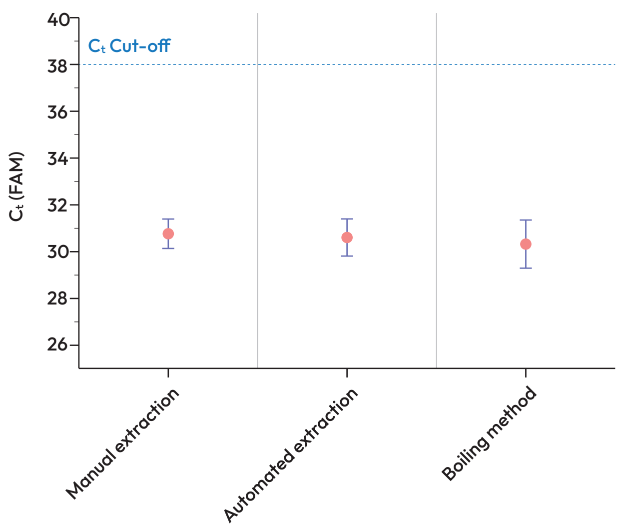


Figure 4 : Reliability and reproducibility of the Mycoplasma Target (FAM) assay under different nucleic acid extraction methods. Identical samples (10 CFU/mL *A. laidlawii* DNA) were tested using manual, automated, and boiling extraction workflows to assess robustness across sample preparation conditions.

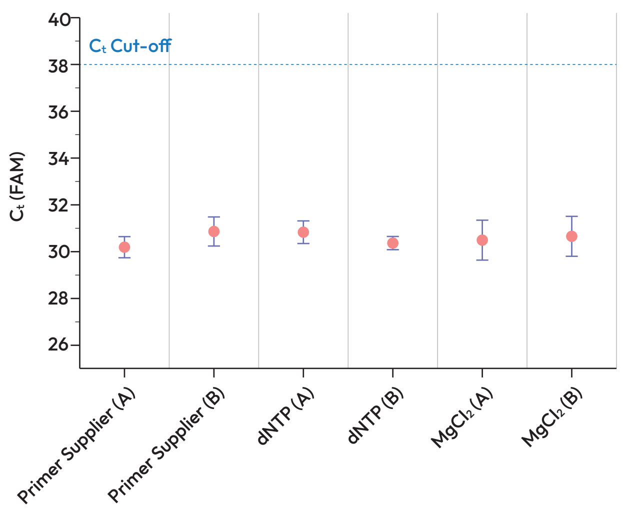


Figure 6 : Reliability and reproducibility of the Mycoplasma Target (FAM) assay under different PCR reagent conditions. Real-time PCR was performed using identical samples (10 CFU/mL *A. laidlawii* DNA) to assess robustness across primer suppliers and varying dNTP and $MgCl_2$ concentrations.

Table 4 : The average Ct values and coefficients of variation (CV, %) were calculated for each condition.

Parameter	Condition	Target Ct (*A.laidlawii*, 10 CFU/mL)				*SD of Ct (FAM)	**CV(%) of Ct (FAM)	Detection rate(%)
		Run 1	Run 2	Run 3	Mean Ct (FAM)			
Real-time Instrument	CFX96	30.52	31.48	30.91	30.97	0.48	1.56	100
	QS5	30.52	30.36	30.51	30.46	0.09	029	100
	FaSTARamp96	26.01	26.05	26.12	26.06	0.06	0.21	100
Extraction Method	Manual extraction	30.43	30.50	31.52	30.82	0.61	1.98	100
	Automated extraction	29.96	31.51	30.51	30.66	0.79	2.56	100
	Boiling method	31.55	29.75	29.87	30.39	1.01	3.31	100
Sample Matrix	Cell Culture (1×10^5 cell/mL)	30.28	30.49	30.11	30.29	0.19	0.63	100
	Cell Culture (1×10^6 cell/mL)	31.19	31.55	30.08	30.94	0.77	2.48	100
	Cell Culture (1×10^7 cell/mL)	30.17	31.50	30.40	30.69	0.71	2.32	100
	FBS	29.62	30.04	31.11	30.26	0.77	2.54	100
	Fresh media (RPMI 1640)	30.69	29.60	29.97	30.09	0.55	1.84	100
PCR Condition	Primer Supplier (A)	30.14	30.73	29.86	30.24	0.44	1.47	100
	Primer Supplier (B)	31.15	31.36	30.20	30.90	0.62	2.00	100
	dNTP (A)	30.91	30.36	31.33	30.87	0.49	1.58	100
	dNTP (B)	30.72	30.26	30.24	30.41	0.27	0.89	100
	$MgCl_2$ (A)	29.72	30.44	31.43	30.53	0.86	2.81	100
	$MgCl_2$ (B)	31.13	31.22	29.73	30.69	0.84	2.72	100

*SD : standard deviation
**CV : coefficient of variation

A CV ≤ 5% was considered acceptable, indicating low variability and high reproducibility.

To evaluate product robustness under minor variations in user- and product-dependent factors, performance testing was conducted across multiple parameters, including PCR instruments (CFX96, FaSTARamp96, QS5), nucleic acid extraction methods (manual, automated, and boiling), primer suppliers (A and B), sample matrices (FBS and cell culture at 1×10^5, 1×10^6, and 1×10^7 cells/mL), and reagent compositions (dNTP conditions and $MgCl_2$ concentrations). Using identical samples containing 10 CFU/mL of *A. laidlawii* genomic DNA, all conditions showed consistent detection, with mean Ct values ranging from 26–30 and 100% positive detection (n=51). Standard deviations were below 1.0 (SD: 0.06–0.86), and coefficients of variation were under 5% (CV: 0.21–2.81%), confirming high reproducibility and reliability. The slightly lower Ct observed on the FaSTARamp96 (mean Ct : 26.01) is considered to result from instrument-specific characteristics compared to the CFX96 and QS5 (Table 4).

Conclusion

Nucleic acid amplification techniques (NAT) based methods for mycoplasma detection represent a significant advancement in quality control practices within the biopharmaceutical and cell therapy industries. The **e-Myco™ VALiD-Q Real-Time PCR Kit (v3.0 PRO)** from iNtRON Bio offers a fully validated and automated platform designed to meet evolving regulatory expectations while addressing product-specific challenges inherent to mycoplasma testing. Recent improvements in sample preparation and assay robustness enable consistent and sensitive detection of mycoplasma across a broad range of biologics, including high-density cell therapies and complex matrices. The system minimizes risks associated with PCR inhibition and matrix interference, maintaining high analytical performance even under the most demanding conditions.

As regulatory authorities in regions such as Europe and the United States increasingly recognize the validity of NAT-based rapid testing methods, the e-Myco™ VALiD-Q Real-Time PCR system has emerged as a practical and efficient alternative to conventional compendial culture methods. Its compatibility with both in-process testing and the use of Internal Control (IC) and External Control (EC), combined with same-day results and minimal hands-on time, provides significant operational advantages.

The integration of the e-Myco™ VALiD-Q Real-Time PCR system into mycoplasma control workflows enhances manufacturing efficiency, ensures product safety, and supports global regulatory submissions and approvals. In conclusion, the adoption of the e-Myco™ VALiD-Q Real-Time PCR Kit (v3.0 PRO) as a rapid, NAT-based real-time detection method represents a forward-looking solution that redefines the standard for mycoplasma testing in biomanufacturing environments.

References

1. European Pharmacopoeia(EP), 11th Edition (2023), 2.6.7
2. United States Pharmacopeia (USP), USP 47 – NF 42 (2024), <63>
3. Japanese Pharmacopoeia (JP), 18th Edition (2021), G3-14-170
4. Korean Standards and Test Methods for Biological Products. Ministry of Food and Drug Safety Notification No. 2022-58

Ordering Information

Application	Product Name	Cat. No.	Size
Extraction	G-spin™ Total DNA Extraction Mini Kit	17045 17046	50 col 200 col
	AutoXT Total gDNA kit	17188-48 17188-96	48 Tests 96 Tests
Detection	e-Myco™ VALiD-Q Real-time PCR Kit (v3.0 PRO)	25247	100 Tests
Prevention	MycoClean™ plus Mycoplasma Prevention Spray	21084	200 mL
Instrument	Miracle-AutoXT Automated Nucleic Acid Extraction System	IMC-NC15 PLUS	1 system
	FaSTARamp96 Real-time PCR System	IMC-FAST-A96	1 system

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