Speeding Up Safety

White Paper

An evaluation of bioMérieux's BIOFIRE®[1] mycoplasma technology for testing cell and gene therapy products

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[1] BIOFIRE® is a registered trademark of bioMérieux, SA.

Complex Therapies Pose Unique Challenges

Introduction

Cell and gene therapy represents the next generation of emerging breakthroughs in biologic therapeutics, delivering transformative hope for patients and cutting-edge solutions to complex medical challenges. Biologics can be engineered to bind with extreme precision, resulting in less off-target interaction, lower toxicity, and reduced side effects compared to traditional small-molecule drugs. These therapies are highly desirable, as they can target complex molecular processes to treat diseases such as cancer or autoimmune disorders. Due to the complex nature of the therapy, often using patient-derived material for manufacturing, a need for rapid release testing presents a challenge.

Traditional Mycoplasma Testing Requires Large Sample Volumes and Long Turn-Around Times

In commercial biopharmaceutical manufacturing, it is imperative to ensure the final drug product is safe, pure, efficacious, and reproducible. One pressing safety concern in biological manufacturing is quick mycoplasma detection to shorten the lab to vein turnaround time. Mycoplasma contamination poses a significant risk to cell and gene therapies, as its presence will compromise the safety and integrity of the final drug product. Therefore, detecting these microscopic threats requires a robust and reliable assay qualification process. Traditional mycoplasma growth detection based compendial methods are widely used because of their established reliability. While effective, these methods have longer turnaround times (14–28 days) than other methods which can prolong delivery of onsite treatment for patients in urgent need. An additional consideration and potential challenge for traditional agar/broth-based mycoplasma detection methods is that these methods require relatively large sample volumes which can be problematic for cell therapies where the availability of patient-derived material is limited.

Introduction of Rapid Testing Methods

From the discovery and study of microorganisms by Robert Hooke and Antoni van Leeuwenhoek (1665–1683) to the work of Louis Pasteur in the mid-19th century and continuing into the twenty-first century, research on microorganisms has greatly advanced our understanding of disease transmission and microorganism contamination. Building on historical foundations of microbial detection, bioMérieux SA, a French biotechnology company, is pioneering rapid mycoplasma testing through its BIOFIRE® Mycoplasma system. bioMérieux continues to develop and validate advanced, rapid testing methods to safeguard therapeutic efficacy and patient safety globally while meeting the expectations of regulatory agencies. The BIOFIRE® Mycoplasma offers a rapid closed system sample-to-answer test designed to detect over 130 mycoplasma species with minimal hands-on time. The test requires minimal operator training, and is compliant with the United States, European Union, and Japan pharmacopoeia (USP, EP, JP). Minaris Advanced Testing has adopted bioMérieux's FDA-cleared BIOFIRE® Mycoplasma system as an option for its clients with the objective of accelerating turnaround time and minimizing sample volume in mycoplasma detection testing.

Overview of Initial Feasibility Study

Minaris Advanced Testing evaluated the BIOFIRE® Mycoplasma technology using a variety of cell and gene therapy-related sample matrices, from cell culture media to final drug product. Samples were tested neat and tested spiked with two concentrations of mycoplasma in colony forming units (CFU): 50 CFUs/mL and 100 CFUs/mL of three strains of viable mycoplasma (i.e. *M. hyorhinis*, *M. orale*, and *M. pneumoniae*). No mycoplasma was detected in any of the neat samples, whereas all spiked samples tested positive for mycoplasma. These results confirmed that the BIOFIRE® Mycoplasma provides a rapid and reliable platform for the testing of mycoplasma for cell and gene therapies in compliance with USP, EP, and JP guidelines. Furthermore, actively growing Jurkat-T cell cultures spiked with four different clinically relevant mycoplasma species (*M. hyorhinis*, *M. orale*, *M. pneumoniae* and *A. laidlawii*) were consistently detected at a concentration of 1 CFU/mL , thereby meeting the guidelines provided in EP 2.6.7 (version 12.2) , Japanese Pharmacopoeia (JP 18 G3) and United States Pharmacopeia (USP) <63> which requires the mycoplasma detection assay sensitivity to detect 10 CFU/mL. This white paper addresses several key aspects of mycoplasma detection, including the challenges associated with current methods and an overview of the BIOFIRE® Mycoplasma system. It outlines the methodology used in the R&D study and method qualification, presents the results and discussion, and concludes with insights and potential future directions for improving mycoplasma detection techniques.

Regulatory Requirements and Traditionally Accepted Methods

Mycoplasma testing of cell and gene therapy products is a regulatory requirement set by the global health authorities (e.g., FDA and EMA). Mycoplasmas are small prokaryotic organisms that lack cell walls and therefore cannot be detected by certain standard methods of microbiology such as gram staining. However, mycoplasmas cause a high risk of contamination in cell cultures and, if present in drug products, can trigger immune reactions and cause serious side effects in patients. Mycoplasma testing is a key component of product safety, ensuring the sterility of the cell and gene therapy product before release for patient treatment.

Mycoplasma testing in biopharmaceutical manufacturing follows stringent regulatory guidelines outlined by the European Pharmacopoeia (EP) Section 2.6.7, The Japanese Pharmacopoeia (JP) <G3-14-170>, and the United States Pharmacopoeia (USP) <63>. The culture-based method remains the standard due to its broad detection capabilities and regulatory acceptance. This method includes a detection element as well as a mycoplasmastasis to evaluate potential inhibition by the product. The standard culture-based method has drawbacks of being both labor-intensive and time-consuming. Testing requires up to 28 days for completion, dedicated laboratory space, and specially trained personnel to perform the analysis.

The "Touchdown" polymerase chain reaction (TD-PCR) offers a faster alternative to standard culture-based methods by using PCR amplification and gel electrophoresis to detect mycoplasma DNA. This method targets a conserved sequence within the mycoplasma genome and provides sensitivity down to 10 copies/mL or approximately 10 colony-forming-units (CFU). While TD-PCR significantly reduces testing time compared to culture-based assays, it is labor-intensive and requires skilled scientists to achieve consistent results.

Thermo Fisher's MycoSEQ™ system is a PCR-based method designed for rapid and sensitive mycoplasma detection, compliant with EP 6.1 Section 2.6.7 guidelines. Capable of detecting 10 copies/mL of mycoplasma DNA in cell culture supernatants or pellets within a single day, MycoSEQ™ provides sensitivity comparable to culture-based methods. However, it poses challenges for cell therapy products in that sample volume requirements tend to be high. Despite being a kit-based system, its complexity in execution and interpretation necessitates highly trained analysts for reliable performance.

In contrast, the BIOFIRE® Mycoplasma system is a closed "lab in the pouch" sample-to-answer PCR system that detects both DNA and RNA. The set up requires approximately two minutes of hands-on time to initiate testing and does not require highly trained personnel or extensive turnaround time to perform. This closed system testing is enabled by the innovative BIOFIRE® Mycoplasma instrument and pouch, which integrates sample handling, reverse transcription, amplification, detection, and analysis into a single-streamlined process.

Scope of Study

To assess if the BIOFIRE® Mycoplasma system is suitable for use as a qualitative test method for detecting the presence of mycoplasma, without significant interference within a wide range of sample matrices, seven different test articles were chosen for the study. The seven test articles included critical raw materials—fetal bovine serum (FBS), TrypLE™ (animal free trypsin alternative), Dulbecco's Modified Eagle Medium (DMEM), and a GFP-containing plasmid — and representative drug products: CAR-T cells, lentiviral vectors (LVV), and adeno-associated virus (AAV). For this study, the 0.2 mL at-line in-process sample protocol was used to evaluate the feasibility of the method. The study examined three Mycoplasma species, introduced at two concentration levels within these representative samples, to assess the sensitivity of mycoplasma detection.

Detection of mycoplasma in cells as well as cell culture media in pre-harvest bulk products during the manufacturing process is crucial for clinical lot release. To detect mycoplasma in active cell cultures, Jurkat cells growing in RPMI media were selected as the representative test article and were pre-spiked with each of four different mycoplasma species (*M. hyorhinis*, *M. orale*, *M. pneumoniae*, and *A. laidlawii*) at a concentration of 10, 1.0 and 0.1 CFU/mL and then processed for mycoplasma detection. The study was designed to determine if the assay was sensitive enough to detect mycoplasma DNA in cell culture as well as cells at the recommended concentration as per regulatory guidelines and to determine the assay Limit of Detection (LOD).

Methodology

Eighteen (18) experimental runs were performed using FBS, TrypLE, DMEM, CAR-T cells, LVV Crude Harvest, AAV, and pSF-AAV-EGFP, as the test articles. Each of the seven (7) test articles was evaluated neat using the BIOFIRE®, followed by eleven (11) runs with spiked live mycoplasma species (*M. hyorhinis*, *M. pneumoniae* and *M. orale*). The generation of test articles, mycoplasma species, and concentration combinations were randomly generated using JMP software.

Similarly, 10 mL of Jurkat-T cell suspension were pre-spiked with three concentrations (10, 1.0 and 0.1 CFU/mL) for each of the live mycoplasma species (*M. hyorhinis*, *M. pneumoniae*, *M. orale* and *A. laidlawii)* in triplicates along with the non-spiked negative control for a total of forty (40) experimental runs. To achieve the desired concentrations, live mycoplasma species were serially diluted using 1x PBS buffered saline (free of calcium and magnesium). From these dilutions, a specified volume from one known concentration was spiked into the test article to achieve the final concentration.

The process of mycoplasma detection begins with pouch preparation, where the BIOFIRE® Mycoplasma pouch, containing the freeze-dried reagents, is rehydrated using the buffer solution, and a small volume of the test article is added using the injection vial that comes with the kit. The pouch is inserted into the system, where a series of automated steps take place. First, mechanical bead beating disrupts cell membranes, if applicable, to release nucleic acids. The system then extracts and purifies the genetic materials from the sample. After nucleic acid extraction, reverse transcription occurs followed by two separate nested steps of multiplex PCR amplification. Detection occurs in real-time with fluorescence-based reporting; data are automatically analyzed by the integral software.

Test Method Procedure

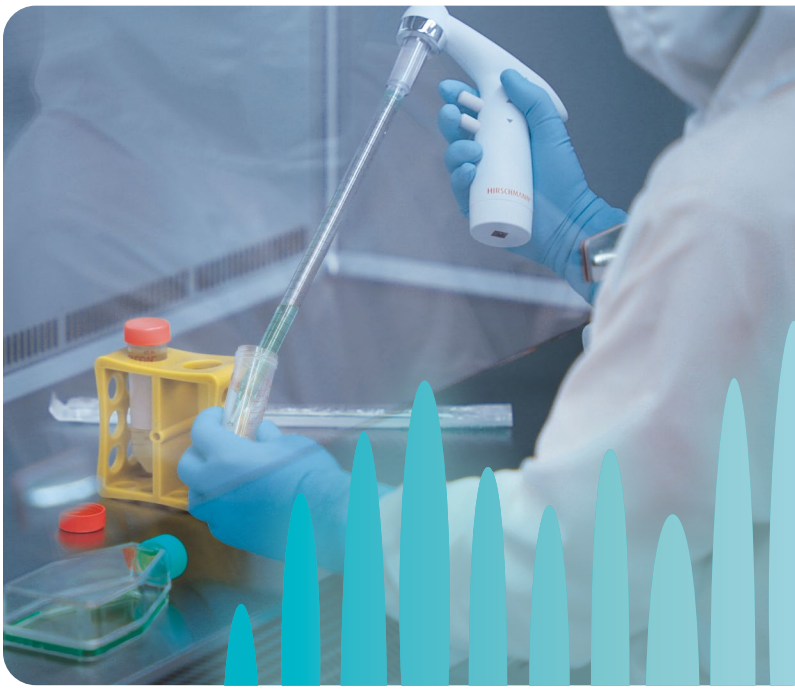
All seven test articles used as matrices along with the mycoplasma stock concentration vials, were removed from freezer storage and thawed at room temperature and processed in a biological safety cabinet along with the BIOFIRE® kit materials in the mycoplasma positive laboratory. For the in-process protocol, live mycoplasma stocks (*M. hyorhinis*, *M. pneumoniae* and *M. orale)* were serially diluted with sterile PBS and appropriate spikes were added to the test articles to form a final concentration of 20 CFU or 10 CFU in a total volume of 200 µL. Non-spiked test articles at a final volume of 0.2 mL were processed simultaneously as negative controls.

For the cell culture method, Jurkat-T cells (1X106 cells) in 10 mL of cell culture media were used as the test article. Each test article was pre-spiked individually with four live Mycoplasma species (*M. pneumoniae*, *M. orale*, *M. hyorhinis*, and *A.laidlawii*) at three different resultant concentrations (10 CFU/mL, 1 CFU/mL & 0.1 CFU/mL) in triplicates. Mycoplasma from cell culture media along with the cells were precipitated using high speed centrifugation. The supernatant was discarded, and the resultant pellet was subsequently analyzed for mycoplasma.

To analyze for the presence of mycoplasma, each test article was pipetted into a new sample injection vial, and one sample buffer packet was added to the same sample injection vial. The sample injection vial was tightly closed and inverted at least three (3) times to mix. The BIOFIRE® pouch was removed from its vacuum sealed package. The hydration injection vial and sample injection vial were both inserted into the pouch to inject the solutions. The appropriate volume of solution was drawn into the pouch via its vacuum mechanism. The pouch was loaded onto the BIOFIRE® instrument. Two operators performed mycoplasma-spiked runs. Operator 1 performed all tasks within the mycoplasma positive laboratory, and Operator 2 worked in the mycoplasma negative laboratory.

Outline of Study Results

Three potential results may be displayed at run completion: 'Mycoplasma Detected' indicates that the sample is positive for mycoplasma contamination; 'Mycoplasma Not Detected' indicates that no mycoplasma was detected in the sample; and 'Invalid' which indicates that the test article and/or internal control run is incomplete, has been aborted, or has otherwise produced an error. No invalid results were observed. All neat samples used as test article matrices received a 'Mycoplasma Not Detected' result, and all spiked samples received a 'Mycoplasma Detected' result. The objective of testing non-spiked samples and spiked samples was to evaluate potential assay matrix interference. The results demonstrated that spiking the samples with three (3) representative mycoplasma species did not adversely affect the performance of the internal controls within the BIOFIRE® pouch. Refer to Table 1 for study results.



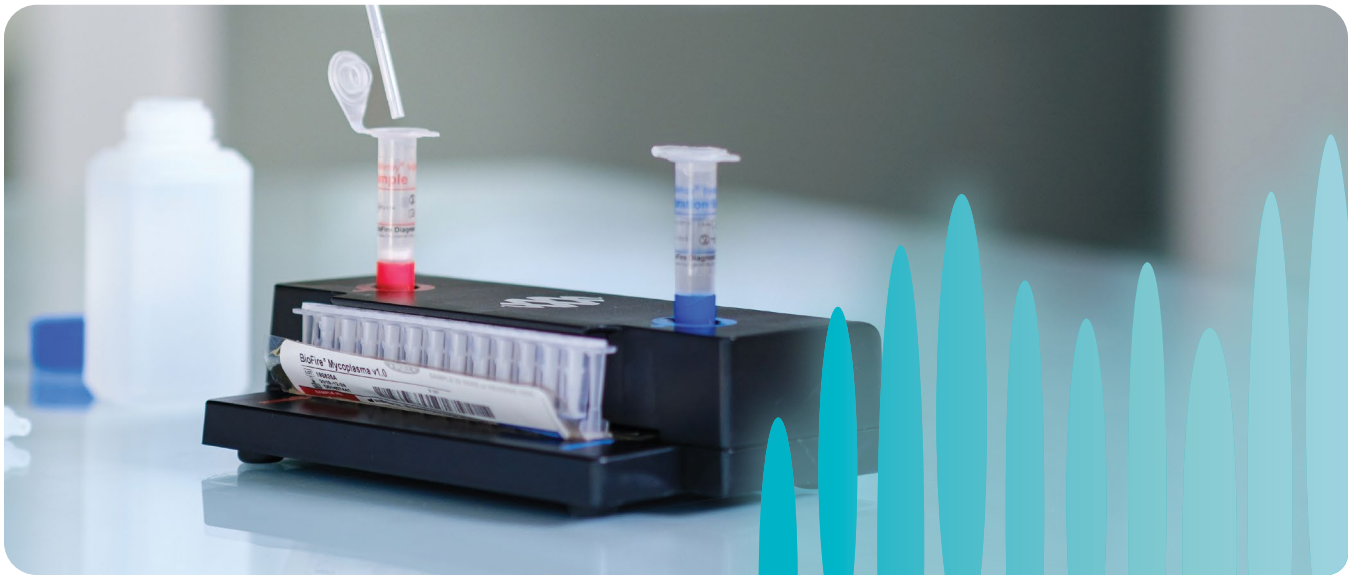
Test Article (TA)	Neat (non-spiked)	*M. hyorhinis*		*M. pneumoniae*		*M. orale*	
		10 CFU	20 CFU	10 CFU	20 CFU	10 CFU	20 CFU
Adeno-Associated Virus	N.D		✓	✓			
Lentiviral Vector	N.D	✓			✓		
pSF-AAV-EGFP	N.D	✓					✓
CAR-T Cells	N.D					✓	
Fetal Bovine Serum	N.D		✓	✓			
Dulbecco's Modified Eagle Medium	N.D					✓	
TrypLE™	N.D						✓

Table 1. R&D Study 1 Experimental Data (N.D – not detected; ✓ – Mycoplasma Detected). 200µL of the TA was spiked with 20 CFU (100 CFU/mL) or 10 CFU (50 CFU/mL) of mycoplasma and tested using BIOFIRE® pouch.

Mycoplasma was successfully detected in every replicate for Jurkat-T cells spiked with 10 CFU/mL of all four mycoplasma species (Table 2). Analysis of the subsequent mycoplasma spike -in dilutions of 1 CFU/mL and 0.1 CFU/mL demonstrates that the assay can detect the presence of mycoplasma at 1 CFU/mL in all tested replicates (Table 2). Thus, the established limit of detection (LOD) for the assay at 1 CFU/mL is ten-fold less than the recommended 10 CFU/mL detection levels in all four clinically relevant mycoplasma species.

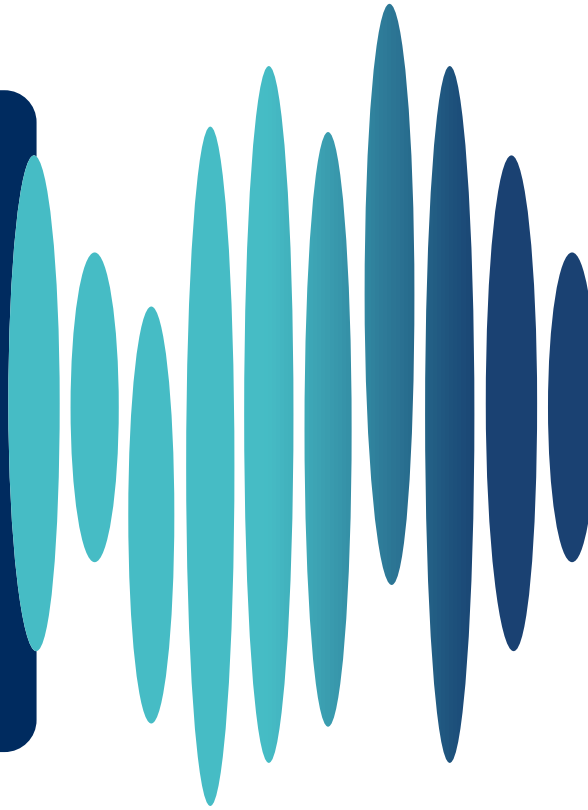
Mycoplasma Test Species	Final Mycoplasma concentration (CFU/mL)	Mycoplasma Detection Rate	Detection (%)	LOD (CFU/mL)
M. pneumoniae	10	3/3	100	1
	1	3/3	100	
	0.1	2/3	66.7	
M. orale	10	3/3	100	1
	1	3/3	100	
	0.1	1/3	33.3	
M. hyorhinis	10	3/3	100	1
	1	3/3	100	
	0.1	0/3	0	
A. laidlawii	10	3/3	100	1
	1	3/3	100	
	0.1	2/3	66.6	
Non-spiked control	N/A	0/4	0	N/A

Table 2: LOD analysis



Conclusion and Recommendations

Based on the findings of this study, the BIOFIRE® Mycoplasma successfully detected mycoplasma across a variety of raw materials and representative cell and gene therapy drug substance/drug products. In active cell cultures which are representative of pre-harvest bulk manufacturing products, BIOFIRE® Mycoplasma successfully detected four different mycoplasma species at 1 CFU/mL, thereby meeting the regulatory requirement for a method with Limit of Detection at 10 CFU/mL. This method was easy to implement and had a shorter turnaround time when compared to current approaches. No instrument failures or invalids occurred, and no samples produced inconclusive results. Therefore, the Minaris Advanced Testing team recognizes the use of the BIOFIRE® Mycoplasma System as an innovative approach to test cell and gene therapies for product release.



Next Steps to Enable Further Adoption

Contract development and manufacturing organizations (CDMOs) have become critical partners for biopharmaceutical companies and help them meet and exceed constantly evolving regulations and market demands. To meet clients' unique goals, CDMOs, and other operational entities must respond in a timely manner to advancement in testing and technology. Switching testing technology, however, presents many challenges, including addressing any skill gaps, evaluating how well the new testing tools integrate into existing processes and systems, conducting a thorough cost- benefit analysis, and importantly, confirming that the new technological approach aligns with regulatory considerations.

As we continue to integrate the BIOFIRE® Mycoplasma System into Minaris' operational workflow and service offerings, the system's reliability, sensitivity, and scalability will be assessed during both release testing and in-process control testing, ensuring it can support a range of applications.

Additionally, future studies will focus on comparing the BIOFIRE® to traditional methods in real-time scenarios, identifying potential advantages based on speed, cost-efficiency, and ease of implementation. Minaris Advanced Testing will assess user feedback, technical integration challenges, and regulatory acceptance, with the goal of refining processes and offering an enhanced testing solution to clients. By leveraging this cutting-edge technology, Minaris Advanced Testing aims to establish a robust framework for future mycoplasma testing that can evolve alongside new therapeutic modalities, contributing to higher efficiency and better patient safety across the biopharmaceutical industry.

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About Minaris Advanced Testing

Minaris Advanced Testing provides multimodality biosafety testing services for cell and gene therapies and biologics, including viral clearance, biosafety testing, product characterization, and GMP analytics to support programs from development through commercial readiness. With a client-first approach, Minaris Advanced Testing delivers efficient, cost-effective testing solutions and a streamlined experience that makes it easier for sponsors to execute studies and advance their programs.

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