

Keywords or Phrases

Mycoplasma detection, magnetic bead-based, DNA extraction, Cyclus® Bead extraction, PCR-based testing

Evaluation of the capability to detect 10 CFU/ mL of Mycoplasma using Cyclus® Bead Extraction in combination with the Microsart® ATMP Mycoplasma Real-time PCR-based detection Kit

Natalie Schmitz¹, Dr. Alexandra Müller-Scholz¹, Gwendolin Auditor¹, Dr. Diana Patzelt²

¹ Sartorius, Goettingen, Germany

² Sartorius, Aubagne, France

Abstract

Mycoplasma testing is a crucial quality parameter for cell therapy products, or other cell culture derived samples. The traditional Mycoplasma test can take up to 28 days to definitively rule out contamination, this time-to-result is insufficient for personalized therapies aiming at treating terminally ill patients. Consequently, there is a strong demand for rapid assays that are growth independent. In response to this demand, we have expanded our portfolio with the Cyclus® Bead Extraction kit, that offers an efficient solution for manual or automated nucleic acid extraction for a wide range of test matrices. This innovative sample prep kit can be combined with Microsart® ATMP Mycoplasma, a Real-time PCR-based Mycoplasma detection for USP and EP conform contamination testing. In this study, we evaluated the capability of this workflow combination to achieve the regulatory required sensitivity for Mycoplasma testing of 10 CFU/ mL in different matrices and for multiple species.

Introduction

Mycoplasma are bacteria that lack a cell wall, making them resistant to many antibiotics and difficult to detect using traditional contamination testing methods. These microorganisms can contaminate cell cultures and cell culture-derived biologicals, affecting the integrity and reliability of biopharmaceutical products. Therefore, rigorous testing for Mycoplasma is essential in the production and development of these products, including vaccines, biologics, and other therapeutic agents derived from cell cultures.

Traditional growth-based techniques for Mycoplasma detection can take up to 28 days, which exceeds the shelf-life of many biologicals or delays treatment for critically ill patients. To address this, Nucleic Acid Amplification Techniques (NAT), such as PCR, have become the standard method for detecting Mycoplasma in cell cultures and cell culture-derived biologicals. NAT methods offer superior sensitivity and specificity, allowing for quick and precise identification of Mycoplasma DNA, ensuring the safety and efficacy of pharmaceutical products. Our existing Microsart® ATMP Mycoplasma kit, along with the new Cyclus® Bead Extraction method, has proven effectiveness for Mycoplasma detection in these critical applications.

However, cell culture testing as part of pharmaceutical quality control can present unique challenges, such as high protein content, complex matrices, and high cell density. These factors can interfere with the accuracy of PCR tests used to detect Mycoplasma contamination, making it harder to identify specific microorganisms, which is crucial for microbiological quality control.

Cyclus® Bead Extraction enhances the capability to extract nucleic acids from an even broader range of sample matrices. This extension allows to handle diverse and challenging samples, ensuring efficient nucleic acid extraction and reliable detection in subsequent PCR amplifications. This extraction method utilizes magnetic beads and is available with both, a manual protocol and an automated DNA

extraction protocol for use with the KingFisher™ Flex device. The automatic extraction process is comprehensively explained in the instruction for use. The bead-based DNA extraction process involves a lysis step, followed by nucleic acid binding to the magnetic beads, three washing steps, and finally the elution of the nucleic acids (Figure 1). Magnetic beads enable efficient binding of even small amounts of nucleic acids while effectively removing potential PCR inhibitors and matrix components that could otherwise reduce PCR sensitivity.

Following the efficient lysis and purification of nucleic acids using the Cyclus® Bead Extraction kit, the extracted DNA serves as the template for the subsequent mycoplasma detection. The Microsart® ATMP Mycoplasma Kit detects mycoplasma contamination by real-time PCR using specific fluorescent probes. It employs two fluorescence channels: FAM™ for the detection of mycoplasma DNA and ROX™ as an internal amplification control to verify PCR efficiency and exclude false-negative results. Each run includes both positive and negative controls to ensure assay validity and result reliability.

Methods

The detection of *Mycoplasma pneumoniae* at a concentration of 10 CFU/mL was evaluated across different sample matrices using the Cyclus® Bead Extraction kit and the Microsart® ATMP Mycoplasma kit. For this purpose lyophilized Microsart® Validation Standards (quantified 10 CFU standards) of the species *Mycoplasma pneumoniae* (SMB95-2014) were rehydrated in 1 mL each of the following matrices:

- Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS)
- avian vaccine (protein-rich matrix)
- 1 mg/mL supercoiled plasmid in Tris buffer
- 1 mg/mL linear DNA in Tris buffer
- 1 mg/mL supercoiled plasmid with known RNA contamination in Tris buffer
- Tris buffer

After an incubation time of 5 minutes, the rehydrated 10 CFU standards were vortexed and used as spiked samples with a known, quantified concentration of 10 CFU/mL. All nucleic acid containing sample matrices were provided by Sartorius Xpress Biologics. To include multiple species in the data set the detection of *Mycoplasma pneumoniae*, *Mycoplasma orale* (SMB95-2012), and *Acholeplasma laidlawii* (SMB95-2018) at a concentration of 10 CFU/mL was evaluated in a matrix of 1 mg/mL supercoiled plasmid in Tris buffer using the Cyclus® Bead Extraction kit and the Microsart® ATMP Mycoplasma kit. Lyophilized Microsart® standards were rehydrated in 1 mL test matrix, incubated for 5 minutes, vortexed and used as spiked samples with a known, quantified concentration of 10 CFU/mL.

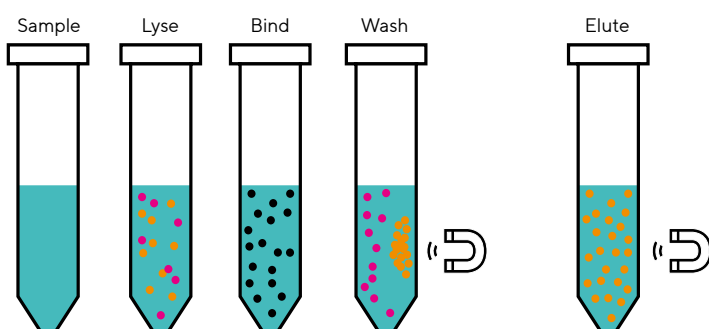


Figure 1: Schematic overview on steps of the Cyclus® Bead Extraction protocol

For both test set ups the samples were extracted together with a Negative Extraction Control (NEC) of the respective matrix without any Mycoplasma spike according to the Cyclus® Bead Extraction (SMB95-6000) instructions for use. 10 µl of each resulting DNA extract were further processed in a Real-time PCR using Microsart® ATMP Mycoplasma (SMB95-1003) following the instructions for use respectively. For thermal cycling and sample analysis the qPCR device qTOWERiris (Analytik Jena) or the QuantStudio 5 (Thermo Fisher) were used.

Results

The detection of *Mycoplasma pneumoniae* at a concentration of 10 CFU/mL was evaluated across different sample matrices using the Cyclus® Bead Extraction kit and the Microsart® ATMP Mycoplasma kit. The results are summarized in Tables 1-4 and Figures 2 and 3. Besides multiple matrices, also multiple species were tested to demonstrate the reliable detection of Mycoplasma species.

Multiple Matrix Testing

The Ct values for 10 CFU/mL *Mycoplasma pneumoniae* in DMEM 5% FBS ranged from 29.0 to 35.9 in the FAM™ channel and 30.1 to 31.7 in the ROX™ channel. The No

Template Control (NTC) and NEC showed no signal in the FAM™ channel as expected, while the internal control detected in ROX™ channel consistently showed Ct values. The Positive Control (PC) demonstrated a Ct value of 23.0 in the FAM™ channel. The detection of the mycoplasma contamination and internal control as well as the expected control results confirmed the assays' sensitivity and specificity (Table 1).

In the avian vaccine matrix, Ct values for 10 CFU/mL *Mycoplasma pneumoniae* ranged from 35.3 to 38.1 in the FAM™ channel, with one sample showing undetected results in the ROX™ channel. In FAM™ positive samples the internal ROX™ control is not relevant. The NTC and NEC controls were negative as expected in the FAM™ channel, while the ROX™ channel had Ct values of 32.9 and 34.2, respectively. The PC showed a Ct value of 23.7 in the FAM™ channel, indicating reliable assay functionality (Table 2).

For samples with high nucleic acid backgrounds, including supercoiled plasmid, linear DNA, and linear DNA with RNA contamination, the FAM™ channel showed Ct values ranging from ~31 to ~33, while the ROX™ channel ranged from 31.8 to 33.8. The NTC and NEC controls were FAM™ negative as expected, with ROX™ channel values of 30.6 and 32.4, respectively. The PC

Sample	Channel	Ct values						
10 CFU/mL <i>M. pneumoniae</i> DMEM 5% FBS	FAM/ ROX	35.9/31.2	30.8/31.7	29.0/31.3	32.4/30.1	31.2/30.5	29.1/30.6	31.7/30.9
Controls	Channel	Ct values						
NTC	FAM/ ROX	Undetected/ 31.0						
NEC	FAM/ ROX	Undetected/ 31.2						
PC	FAM/ ROX	23.0/ 31.2						

Table 1: Results of 10 CFU/mL *Mycoplasma pneumoniae* detection in DMEM 5% FBS extracted using the Cyclus® Bead Extraction kit and measured with the Microsart® ATMP Mycoplasma kit

Sample	Channel	Ct values			
10 CFU/mL <i>M. pneumoniae</i> avian vaccine	FAM/ ROX	35.3/ 36.3	36.2/ 36.4	38.1/ 34.9	35.3/ Undetected
Controls	Channel	Ct values			
NTC	FAM/ ROX	Undetected/ 32.9			
NEC	FAM/ ROX	Undetected/ 34.2			
PC	FAM/ ROX	23.7/ Undetected			

Table 2: Results of 10 CFU/mL *Mycoplasma pneumoniae* detection in high protein background avian vaccine extracted using the Cyclus® Bead Extraction kit and measured with the Microsart® ATMP Mycoplasma kit

Sample	Channel	Ct values			
10 CFU/mL <i>M. pneumoniae</i> 1 mg/mL supercoiled Plasmid (Tris buffer)	FAM/ ROX	31.8/ 33.8	31.8/ 33.0	32.2/ 33.1	31.9/ 32.0
10 CFU/mL <i>M. pneumoniae</i> 1 mg/mL linear DNA with RNA contamination (Tris buffer)	FAM/ ROX	33.8/ 33.7	33.1/ 32.7	33.7/ 32.4	32.8/ 31.8
10 CFU/mL <i>M. pneumoniae</i> 1 mg/mL linear DNA (Tris buffer)	FAM/ ROX	35.1/ 33.1	33.1/ 32.8	33.5/ 33.0	32.9/ 33.2
10 CFU/mL <i>M. pneumoniae</i> in Tris buffer	FAM/ ROX	32.6/ 32.7	32.1/ 32.6	32.6/ 32.6	32.4/ 33.4
Controls	Channel	Ct values			
NTC	FAM/ ROX	Undetected/ 30.6			
NEC	FAM/ ROX	Undetected/ 32.4			
PC	FAM/ ROX	24.5/ 30.3			

Table 3: Results of 10 CFU/mL *Mycoplasma pneumoniae* detection in high nucleic acid backgrounds extracted using the Cyclus® Bead Extraction kit and measured with the Microsart® ATMP Mycoplasma kit

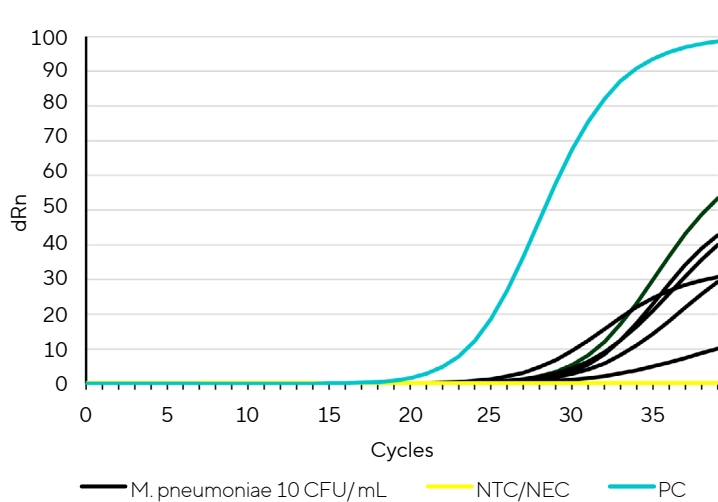


Figure 2: PCR results of the FAM™ channel for 10 CFU/mL *M. pneumoniae* rehydrated in DMEM 5% FBS, extracted using the Cyclus® Bead Extraction kit and amplified using the Microsart® ATMP Mycoplasma kit with the qTOWERiris (Analytik Jena), as well as Negative Extraction Control, No Template Control and Positive Control.

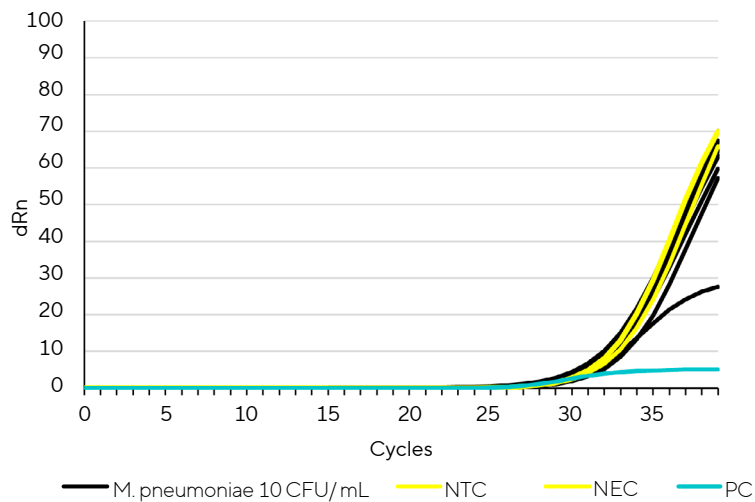


Figure 3: PCR results of the ROX™ channel for 10 CFU/mL *M. pneumoniae* rehydrated in DMEM 5% FBS, extracted using the Cyclus® Bead Extraction kit and amplified using the Microsart® ATMP Mycoplasma kit with the qTOWERiris (Analytik Jena), as well as Negative Extraction Control, No Template Control and Positive Control.

demonstrated a Ct value of 24.5 in the FAM™ channel, confirming the assay's functionality (Table 3).

Exemplary figures of the PCR amplification are shown in Figure 1 for the FAM™ channel detection and in Figure 2 for the ROX™ channel.

The combination of Cyclus® Bead Extraction and Microsart® ATMP Mycoplasma successfully detected *Mycoplasma pneumoniae* in all tested matrices.

Multiple Mycoplasma Species Testing

The detection of *Mycoplasma pneumoniae*, *Mycoplasma orale*, and *Acholeplasma laidlawii* at a concentration of 10 CFU/mL was evaluated in a matrix of 1 mg/mL supercoiled plasmid in Tris buffer using the Cyclus® Bead Extraction kit and the Microsart® ATMP Mycoplasma kit.

For *Mycoplasma pneumoniae*, the Ct values ranged from 31.8 to 32.2 in the FAM™ channel and from 32.0 to 33.8 in the ROX™ channel. *Mycoplasma orale* showed Ct values ranging from 34.0 to 38.8 in the FAM™ channel and from 31.9 to 32.7 in the ROX™ channel. *Acholeplasma laidlawii* had Ct values ranging from 32.8 to 34.3 in the FAM™ channel and from 31.8 to 32.5 in the ROX™ channel.

The NTC and NEC showed no detection in the FAM™ channel, while the ROX™ channel had Ct values of 32.0 and 30.1, respectively. The PC showed Ct values ranging from 23.5 to 24.5 in the FAM™ channel and 29.4 in the ROX™ channel, confirming the assay's sensitivity and specificity.

These results demonstrate the capability of Cyclus® Bead Extraction and Microsart® ATMP Mycoplasma to reliably detect various Mycoplasma species in a complex nucleic acid matrix.

Sample	Channel	Ct values			
10 CFU/ mL <i>M. pneumoniae</i> in 1 mg/ mL supercoiled Plasmid (Tris buffer)	FAM/ ROX	31.8/ 33.8	31.8/ 33.0	32.2/ 33.1	31.9/ 32.0
10 CFU/ mL <i>M. orale</i> in 1 mg/ mL supercoiled Plasmid (Tris buffer)	FAM/ ROX	38.8/ 31.9	34.6/ 32.7	35.2/ 32.5	34.0/ 32.0
10 CFU/ mL <i>A. laidlawii</i> in 1 mg/ mL supercoiled Plasmid (Tris buffer)	FAM/ ROX	33.4/ 32.5	33.4/ 31.8	32.8/ 32.0	34.3/ 32.1
Controls	Channel	Ct values			
NTC	FAM/ ROX	Undetected/ 32.0			
NEC	FAM/ ROX	Undetected/ 30.1			
PC	FAM/ ROX	23.5-24.5/ 29.4			

Table 4: Results of 10 CFU/ mL *Mycoplasma pneumoniae*, *Mycoplasma orale* and *Acholeoplasma laidlawii* detection in high nucleic acid backgrounds extracted using the Cyclus® Bead Extraction kit and measured with the Microsart® ATMP Mycoplasma kit

Discussion

The assays conducted in this study were successful, as evidenced by the control results, confirming the correct execution of all procedures. The detection of *Mycoplasma pneumoniae* spikes across various matrices was achieved reliably, demonstrating the robustness of the Cyclus® Bead Extraction and Microsart® ATMP Mycoplasma kits.

Furthermore, the detection of different *Mycoplasma* species, including *Mycoplasma pneumoniae*, *Mycoplasma orale*, and *Acholeoplasma laidlawii*, was accomplished with high reliability, even in a complex matrix at a concentration of 10 CFU/mL. This underscores the versatility and effectiveness of the kits in handling various species and matrix complexities. Consistency and reliability can be improved even more by applying the automatic extraction using the KingFisher™ Flex device.

To address the challenges posed by more complex matrices, minor protocol adjustments were implemented. These adjustments are a standard practice in assay optimization, ensuring accurate detection in diverse sample environments. The PCR team at Sartorius is readily available to assist with validation support, protocol modifications and technical consultation through the email address PCR@sartorius.com.

Conclusion


Cyclus® Bead Extraction expands Sartorius PCR portfolio with an efficient manual and automatic nucleic acids extraction kit to detect 10 CFU/ mL *Mycoplasma* reliably.

Germany

Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0

USA

Sartorius Corporation
3874 Research Park Dr.
Ann Arbor, MI 48108
Phone +1 734 769 1600

 For further information, visit
www.sartorius.com