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A New Real-Time RT-PCR-Based Mycoplasma Detection Kit to Meet the Current Revision of Ph. Eur. Chapter 2.6.7 and USP <77> Draft

Michael Joyce[1], Natalie Schmitz[2], Robert Hertel[2], Alexandra Mueller-Scholz[2], Diana Patzelt[3]

[1] BioRestorative Therapies, Inc., Melville, NY, USA
[2] Sartorius, Goettingen, Germany
[3] Sartorius, Aubagne, France

For further information or support contact: PCR@sartorius.com

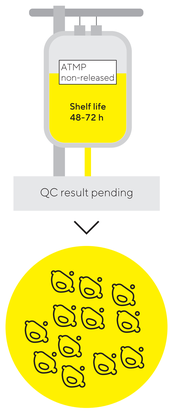
Abstract

Mycoplasma testing is a critical quality attribute (CQA) of cellular therapeutics since microbial contamination of cell therapy products can potentially result in the recipients' deaths. The compendial Mycoplasma test takes up to 28 days before a contamination can be ruled out with certainty, which is too long for autologous cell therapies intended to treat terminally ill patients. As a result, there is a high demand for growth-independent rapid assays.

Currently the regulatory landscape for NAT based Mycoplasma detection is changing, USP <77> is drafted and Ph. Eur. chapter 2.6.7 under revision, setting new requirements on the detection capability of a respective assay. Therefore, a detection system with a highly efficient automated or manual DNA extraction protocol followed by a Real-time RT-PCR assay has been developed. In this study we challenged our current products with complex matrixes and tested the benefit of a newly implemented extraction method to be able to meet the upcoming regulatory versions Ph. Eur. chapter 2.6.7 and USP <77>.

1. Advanced Therapy Medicinal Products (ATMPs)

Advanced Therapy Medicinal Products (ATMPs) are innovative medicines that utilize gene, cell, or tissue-based technologies to offer new treatments for severe and life-threatening conditions. These therapies promise to revolutionize modern medicine by providing targeted and personalized treatment options. However, producing ATMPs involves stringent regulatory requirements to ensure safety and efficacy. ATMPs come with various conditions such as high protein content or cell background. For example, a high cell density can interfere with the sensitivity and specificity of PCR assays designed for Mycoplasma contamination detection, making it difficult to accurately detect targeted microorganisms which is of great importance for microbiological quality control.



2. Mycoplasma Detection Based on Nucleic Acid Amplification

Mycoplasma are bacteria lacking a cell wall, making them resistant to many antibiotics and hard to detect with traditional methods. Additionally traditional growth-based methods take up to 28 days which by far exceeds the shelf-life of many ATMPs or extends the vein-to-vein time for terminally ill patients. Nucleic Acid Amplification Techniques (NAT), such as PCR, have become the gold standard for Mycoplasma testing in ATMPs. NAT methods offer high sensitivity and specificity, enabling rapid and accurate detection of Mycoplasma DNA. Our Microsart® ATMP Mycoplasma kit, in combination with the Microsart® AMP extraction, has successfully demonstrated its efficacy for Mycoplasma detection in ATMPs.

3. Managing High Cell Density Background

BioRestorative Therapies, Inc. (BRTX) develops therapeutic products using cell and tissue protocols, primarily involving adult stem cells. BRTX is in the progress of validating Sartorius' current kit solution for their human bone marrow MSCs (26M cells/mL) matrix. After completion of the validation work BRTX will then be seeking approval from the FDA to use Sartorius's mycoplasma detection assay within their ongoing phase-II clinical trial.

The MSC matrix was utilized to rehydrate lyophilized 10 CFU standards (Microsart® Validation Standards, Sartorius) of four different species (*Mycoplasma orale, Mycoplasma fermentans, Mycoplasma pneumoniae* and *Acheloplasma laidlawii*) and was then used as an input for the Microsart® AMP extraction kit followed by Real-time PCR detection using the Microsart® ATMP Mycoplasma kit. By the integration of minor changes to the extraction protocol, which was supported by the PCR team from Sartorius, all four species were reliably detected in all replicates (Figure 1, Table 1). Two main adjustments led to the successful usage of the kit solution for such high cell density matrices:

To avoid the saturation of the silica matrix by other nucleic acids than the Mycoplasma spike, for example RNA, which is released by fresh lysed cells, an RNAse A treatment was introduced into the protocol. Furthermore, adaption of some centrifugation steps was part of the protocol optimization.

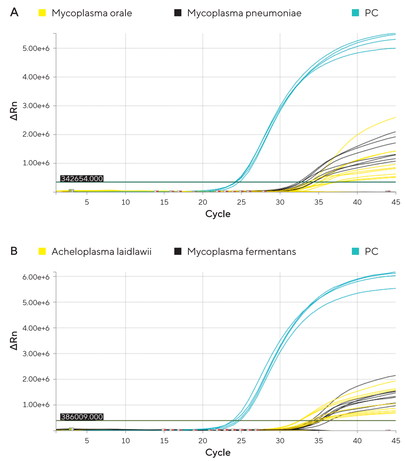


Figure 1: *Amplification curves after extracting 10 CFU of Mycoplasma orale, Mycoplasma pneumoniae (A), Mycoplasma fermentans, and Acheloplasma laidalawii (B) by using using the Microsart® AMP extraction kit (optimized protocol) with subsequent Real-time PCR analysis (Microsart® ATMP Mycoplasma kit)*

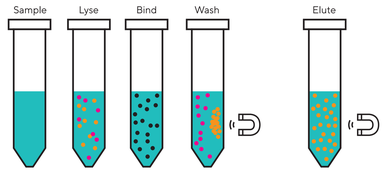
Table 1: *Generated Ct values after extracting 10 CFU of Mycoplasma orale, Mycoplasma fermentans, Mycoplasma pneumoniae and Acheloplasma laidalawii by using the Microsart® AMP extraction kit (optimized protocol) with subsequent Real-time PCR analysis (Microsart® ATMP Mycoplasma kit)*

Species	Mycoplasma spikes								NEC1	NEC2	NTC1	NTC2	PC2	PC2
Mycoplasma orale	31.5	32.3	31.0	31.6	29.4	31.7	33.5	31.7	Undetermined	Undetermined	Undetermined	Undetermined	24.2	24.4
Mycoplasma pneumoniae	32.8	33.3	32.4	34.4	33.7	34.3	32.8	33.7	Undetermined	Undetermined	Undetermined	Undetermined	24.3	24.8
Mycoplasma fermentans	36.5	34.0	34.9	35.0	34.6	34.3	35.0	35.4	Undetermined	Undetermined	Undetermined	Undetermined	23.9	24.2
Acholeplasma laidlawii	34.4	32.5	32.6	33.9	34.9	32.6	35.0	35.6	Undetermined	Undetermined	Undetermined	Undetermined	24.5	24.8

4. Increasing Sensitivity Using a New Extraction Method

Since the need for more sensitive assays and kit solutions is rapidly growing with the revision of important regulatory guidelines and new created chapters, like USP <77> and Ph. Eur. chapter 2.6.7., a combination of the currently available Microsart® ATMP Mycoplasma kit with the planned new generation extraction kit was tested. This combination already revealed increased sensitivity in the detection of 10 CFU/ mL *Acheloplasma laidlawii.*

The new extraction method will be based on magnetic beads and comes with a manual protocol as well as with a protocol for automated DNA extraction. Bead-based DNA extraction includes a lysis step followed by the binding of the DNA to the magnetic beads, a washing step and lastly the elution of the target DNA. Magnetic beads allow a strong binding of minimal DNA amounts and very efficient removing of inhibitor and matrix components which in the end potentially impair PCR sensitivity.



Taking a closer look on the amplification curves generated for 10 CFU/ mL *Acheloplasma laidlawii* using our current Microsart® AMP Extraction kit and the new developed bead-based extraction kit, not only the Ct values were lower but also the fluorescent signal intensity was increased when using the bead-based extraction method arguing on the one hand for a better recovery of the Mycoplasma spike and on the other hand revealing higher PCR functionality due to efficient removal of sample matrix and kit components (Figure 2, Table 2).

Figure 2: *Amplification curves after extracting 10 CFU of Acheloplasma laidalawii by using a spin column-based and bead-based extraction method with subsequent analysis using the Microsart® ATMP Mycoplasma kit.*

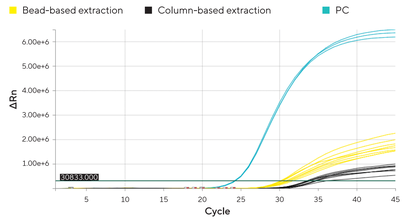
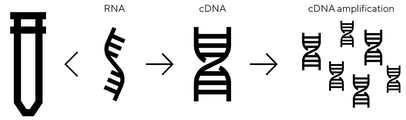


Table 2: *Generated Ct values after extracting 10 CFU of Acheloplasma laidlawii by using a column-based and bead-based extraction method with subsequent analysis using Microsart® ATMP Mycoplasma Real-time PCR.*

	Acheloplasma laidlawii 10 CFU/ mL								NEC (DMEM, 10% FBS)	PC	NTC
Bead-based extraction	31.1	31.1	31.8	31.4	30.9	30.2	30.4	30.3	Undetermined (4/4)	Positive (3/3)	Undetermined (3/3)
Spin Column-based extraction	34.0	33.8	34.6	34.7	33.5	33.7	36.3	34.9	Undetermined (4/4)		

5. Real-time RT-PCR Based Mycoplasma Detection

Regulatory guidelines request a high sensitivity of PCR kits for Mycoplasma contamination detection, with a rising trend while DNA targets are only available in organisms on low copy levels. This is the reason why traditional DNA-based PCR is slowly reaching its limits when trying to keep robustness and reliability of the detection. Real-time Reverse Transcriptase PCR offers a smart solution to overcome this problem. Every gene which is detectable on DNA-level is also available as a transcript within the target organism. Especially the 16S rRNA region, a highly conserved rRNA operon, which is targeted for Mycoplasma detection has multiple RNA copies within one cell. The occurrence of multiple targets on RNA-level facilitates the detection of lower cell numbers with PCR. A reverse transcription polymerase makes the RNA copies available as cDNA targets and therefore multiplies the available PCR targets compared to a basic DNA-based PCR detection. It is true that this approach does not allow any quantitative interpretations of the PCR results since the RNA copy number of the 16S rRNA gene is highly flexible, but a quantitative output is not necessary when it comes to quality control questions which require a Yes or No answer. This approach is particularly simple because the reverse transcription is already implemented within the PCR reaction mix.



We are coming from an extremely effective kit solution going to an even more sensitive approach. The newly developed Mycoplasma detection Kit including a magnetic bead-based extraction, and a Real-time RT-PCR assay will meet the current revision of Ph. Eur. chapter 2.6.7 and USP <77> Draft while still focusing on time to result and convenience.