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Rapid, Real-Time PCR-Based Detection of Microbial Contaminations in High Cell Density Jurkat-, HPBMC- and CHO- Cultures using Microsart® ATMP Kits

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Abstract

In this study, we used Sartorius Real-time PCR kits for the rapid detection of bacteria, fungi, and mycoplasma to establish that these assays can detect microbial contaminants, even in the presence of high cell backgrounds, ranging from 10 to 40 million cells per milliliter (cells/mL). We observed that the cell density limit and assay robustness depend on cell types and media compositions. The detection limits for bacteria, fungi, and mycoplasma in Jurkat cells, HPBMC and CHO cells varies between 10 and 25 x 10^6 cells/mL.

This study clearly demonstrates that Sartorius Microsart® ATMP kits tolerate higher cell density, still reaching the required sensitivity criteria (≤ 99 CFU, or colony forming units, for bacteria and fungi and ≤ 10 CFU for mycoplasma). The study also demonstrates the capability of Microsart® ATMP kits to sensitively detect microorganisms, even in the presence of high-density cell cultures, and thus contributes to risk-reduction and patient safety of cell therapy products.

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Introduction

Sterility testing is a critical component of the release testing for any cell therapy product since microbial contamination of cell therapy products can potentially kill recipients. The current compendial sterility test for most bacteria takes 14 days and 28 days for mycoplasma testing before contamination can be ruled out with certainty[1,2,3]. However, time-to result is an important attribute of testing or short shelf-life cellular therapeutics, especially for autologous cell therapies intended for terminally ill patients.

As a result, growth-independent rapid assays are in increasing demand. Therefore, to fulfill this demand, we developed and comprehensively validated a highly sensitive and broad range microbial detection system, consisting of an efficient Microsart® ATMP Extraction DNA isolation protocol, and followed by a real-time PCR assay using the Microsart® ATMP Bacteria/Fungi/Mycoplasma kit. *In-silico* sequence alignment analysis demonstrated that the Microsart® ATMP Bacteria/Fungi/Mycoplasma assays detect a broad range of microorganisms, including species difficult to detect by classical culture methods, as well as dormant contaminants. In addition, the typically very high cell densities of cellular therapeutics can pose challenges to the sample preparation used in rapid, nucleotide amplification (NAT)-based assays.

In this study, we assessed the detection capability of Microsart® ATMP Extraction, combined with Microsart® ATMP Bacteria/Fungi/Mycoplasma assays, in high-density cell cultures. We used Human Peripheral Blood Mononuclear Cells (HPBMC), Jurkat cells, and Chinese Hamster Ovary (CHO) cells as representative background matrices. We chose HPBMC and Jurkat cells lines because the product was developed primarily for QC testing of ATMPs, and therefore, the Real-time PCR detection kits mainly target applications with human cell lines. We chose CHO cells because they can be cultivated in high densities, making them valuable for tests with very high cell densities[4].

Methods

Preparation of eukaryotic cell samples
Jurkat cells were grown in T-flasks in RPMI medium (RPMI 1640, Thermo Fisher/Gibco), supplemented with 2 mM GlutaMAX (Thermo Fisher/Gibco), 10 % fetal calf serum (Thermo Fisher/Gibco), 1 mg/mL Penicillin, and 104 U Streptomycin (Thermo Fisher/Gibco). Media was exchanged every three to four days. Jurkat cell density was counted with a Cedex HiRes analyzer (Roche).

CHO cells were diluted with Dulbecco's modified Eagle's medium (DMEM) to required concentrations. HPBMC and Jurkat cells were further concentrated by centrifugation for 10 min at 200 x g or 300 x g, and supernatant discarded until the required concentration was reached. Cell pellet was resolved gently.

To prepare cell culture samples with defined microbial spikes, Microsart® Validation Standards, which contain a specific number of CFU of inactivated bacteria or mycoplasma cells, were used according to the instructions[5,6]. Validation standards for *Bacillus subtilis, Pseudomonas aeruginosa, Kocuria rhizophila, Candida albicans, Mycoplasma arginini, Mycoplasma orale* and *Mycoplasma s ynoviae* were used in this study. These standards contain 99 CFU of lyophilized, inactivated bacterial particles or 10 CFU lyophilized, inactivated mycoplasma particles. For fungal contaminations, the EZ-CFU™ standards from Microbiologics® with a spike level of 50 CFU were used. The working suspension was prepared according to the EZ-CFU™ protocol (one CFU pellet, 1:20 diluted)[7].

DNA extraction
DNA was extracted with the Sartorius Microsart® ATMP Extraction kit, suitable for DNA isolation of gram-positive and gram-negative bacteria, fungi, and mycoplasma prior to PCR testing[8]. According to the protocol, 1 mL sample material was added to a DNA free 1.5 mL tube. The tube was centrifuged for 15 min at 16,200 g. Supernatant was discarded, then 500 µL Lysis Buffer added to the pellet. To enable better lysis, the pellet was dissolved with a filter tip or by vortexing. After vigorously vortexing for 30 s, the sample was incubated for 10 min at 80° C (combined with shaking at 1,500 rpm) and centrifuged for 10 min at 16,200 g. Supernatant was discarded, 100 µL Suspension Buffer added, and the tube vortexed vigorously for 30 s. DNA was used for PCR directly.

Quantitative real time polymerase chain reaction (qPCR)
For PCR testing, the Sartorius Microsart® ATMP Bacteria, Microsart® ATMP Fungi, or Microsart® ATMP Mycoplasma kits were used[9,10,11]. These PCR kits use FAM™ and ROX™ fluorescence dyes for real-time detection. The dyes are fused to ssDNA probes, which are complementary to the amplified target DNA (FAM™) or the Internal Inhibition Control (ROX™). The use of these TaqMan® probes enhances the specificity of the assay.

The lyophilized Bacteria/Fungi/Mycoplasma Mix, the Internal Control, and Positive Control were centrifuged briefly to collect the lyophilized material on the bottom of each tube. Reagents were rehydrated by adding 390 µL Rehydration Buffer to the Mastermix (MM), 800 µL PCR grade water to the Internal Control, and 300 µL to the Positive Control. The reagents were incubated for 5 min and briefly vortexed. 26 µL Internal Control was added to the 390 µL MM. Afterwards, the PCR reaction mix was prepared by adding 10 µL sample DNA extract, PCR grade water (no template control), or Positive Control to 15 µL MM in a PCR reaction tube. A total reaction volume of 25 µL per sample was amplified according to the following thermal protocol:

1. 95° C for 3 min
2. 95° C for 30 s
3. 55° C for 30 s
4. 60° C for 45 s

Steps 2 to 4 were repeated 39 times.

Fluorescence of ROX™ and FAM™ was measured during the elongation in step 4.

For most experiments, all pipetting steps were conducted inside a closed glovebox with airlock to avoid contaminations. The BIO-RAD PCR cycler CFX96 Deep Well cycler instrument was used, data were analyzed with the Bio-Rad CFX Manager 3.1 software. Experiments that were conducted without the glovebox, were performed at a sterile bench and with a Stratagene Mx3005p PCR cycler. Data were analyzed with the MxPro software. Ct threshold and baseline were manually adjusted for ROX™ and FAM™ in both software systems. The baseline start was set where fluorescence signal levelled off at a constant level. The baseline end was set before the fluorescence signal of the positive control increased. The threshold was set to a tenth of the mean maximum fluorescence of the no template controls (for ROX™) or the Positive Control (for FAM™) for the bacteria and fungi PCR. Resulting Ct values < 40 were considered positive, CT values > 40 were considered negative. Automatic software settings (baseline and threshold) were used for Mycoplasma detection using the Stratagene Mx3005p PCR cycler.

Results

As summarized in Table 1, we detected 10 CFU *M. arginini* or *M. orale* spikes or 99 CFU of *B. subtilis* in a background of 15 to 19 million CHO cells per ml, using Microsart® Validation Standards. In the presence of 25 million Jurkat cells per ml, we detected 99 CFU of *K. rhizophila* and in presence of up to 20 million Jurkat cells per ml, we detected 50 CFU of *C. albicans*. We did not detect 10 CFU of *M. orale* or *M. synoviae* in the presence of Jurkat cells, due to PCR inhibition.

In up to 10 million HPBM cells per ml, we detected 99 CFU of *K. rhizophila*. In a background of 15 and 19 million cells per ml, we detected 10 CFU of *M. orale* and *M. arginini*. In presence of 20 and 25 million HPBM cells per ml, we detected 99 CFU of *P. aeruginosa*.

Detection limits in different cell types

Cell Type Background	Microorganism Spike	Background Cells /mL (In 10^6)	Detection
CHO	99 CFU *B. subtilis*	19.0	Successful
CHO	10 CFU *M. arginini*	15.0 and 15.6 (two individual experiments)	Successful
CHO	10 CFU *M. orale*	15.5 and 16.3 (two individual experiments)	Successful
Jurkat	99 CFU *K. rhizophila*	10 to 40	Successful up to 25×10^6 c/mL
Jurkat	50 CFU *C. albicans*	10 to 40	Successful up to 20×10^6 c/mL
Jurkat	10 CFU *M. orale*	10 to 40	Not successful: PCR inhibition > 15×10^6 c/mL No detection of Mycoplasma spike
Jurkat	10 CFU *M. synoviae*	10 to 35	Not successful: Partial PCR inhibition No detection of Mycoplasma spike
HPBMC	99 CFU *K. rhizophila*	10 to 40	Successful only up to 10×10^6 c/mL
HPBMC	10 CFU *M. arginini*	15.0	Successful
HPBMC	10 CFU *M. orale*	19.1	Successful
HPBMC	99 CFU *P. aeruginosa*	20 and 25	Successful

Table 1: Results of Real-Time PCR detection of respective microbial spikes in varying cell types with respective cell densities.

Conclusion

Short shelf-life therapeutical products require rapid, alternative methods for sterility testing to rule out contamination before administration. Rapid detection methods, such as Real-time PCR-based approaches, contribute to patient safety; however, the typically very high densities of cellular therapeutics pose challenges to sample preparation protocols. Here, we tested the tolerance of Microsart® ATMP kits to different cell densities and cell types.

We found that the cell density limits and assay robustness varied, depending on cell type and media compositions. While the detection limit in Jurkat cells seems to be around 20 to 25 x 10^6 cells/mL for fungi and bacteria, the PCR reaction itself was inhibited at lower cell densities for the mycoplasma PCR. The limit for HPBMC varies between 10 and 25 x 10^6 cells/mL for bacteria, fungi, and mycoplasma detection. We successfully detected mycoplasma and bacteria in the presence of about 15 to 19 million CHO cells per ml.

The performance of these kits was validated using a maximum cell concentration of 10^6 cells/ml; however, our study demonstrates that even higher cell numbers can be processed without losing sensitivity. The maximum cell number for testing can vary according to specific characteristics of the sample (e.g. medium, cell type) and may require adaptation of the procedure to the specific matrices.

Rapid, sensitive, and robust detection of bacteria, fungi, and mycoplasma in the presence of a dense cell background requires an efficient lysis step, followed by a suitable DNA isolation and a robust PCR assay as offered by Microsart® ATMP kits.

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