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Application Note

Monitoring Microbial Contamination of Mammalian Cell Cultures with the Milliflex® Rapid System 2.0

Milliflex® Rapid System 2.0 is used by manufacturers to monitor their processes and the final drug for microbial contamination. The rapid detection system is based on membrane filtration of the sample to capture contained microorganisms and to subsequently detect any viable contaminants by adenosine triphosphate (ATP) bioluminescence. However, mammalian cells also contain ATP. Where these are used in bioproduction processes or where patient or donor cells are cultured for cell therapies, the mammalian ATP can therefore result in bioluminescence and thus in false positive counts.

A cell lysis buffer was previously developed to selectively lyse the mammalian cells and remove the mammalian ATP during sample preparation, filtration, and rinsing. However, one ingredient is now considered a Substance of Very High Concern (SVHC) by the European Chemicals Agency (ECHA). This has led to the need to reformulate the lysis buffer.

This protocol shows how the replacement buffer can efficiently remove the signal caused by mammalian ATP and thus improve the selectiveness of microbial contaminant detection in filtrable samples containing mammalian cells using the Milliflex® Rapid System 2.0.

Introduction

Mammalian cells are widely used in the biopharmaceutical industry to produce complex processed molecules such as recombinant proteins or monoclonal antibodies. To ensure patient safety and minimize the risk that the end product must be discarded, cell culture batches as well as samples from downstream processes must be thoroughly controlled for their microbial contamination.

Cell culture media are an ideal environment for microbial growth, especially when the end product does not allow the use of antibiotics. Also, the recent development of cancer cell therapies using, for example, CAR-T cells is posing new challenges to microbial monitoring due to the short shelf-life of these cell-based therapeutics.

Chapter 10 Quality Control of the "The EU GMP Annex 1 "Manufacture of Sterile Medicinal Products" states that, when manufacturing short-life products, rapid/ alternative methods should be considered for the detection of contaminant microbes. The USP general information chapter <1071> on Rapid Microbial Tests for Release of Sterile Short-Life Products states that “for a rapid microbial test for the release of sterile short-life products, an enrichment culture either in liquid media to reach a threshold ATP level or on a membrane filter on solid media for the formation of microbial colonies could be used with an incubation time of 2–7 days”.

Milliflex® Rapid System 2.0 allows manufactures to react earlier in case of microbial contamination, thus improving process and final product control. The detection method used by the rapid detection system is based on the well-established, proven technologies of membrane filtration and ATP bioluminescence. However, if samples contain mammalian cells, the intracellular ATP from such cells captured on the membrane also reacts with the bioluminescence reagents, resulting in non-specific background signal. This can be an issue particularly if incubation is short because fast-growing microorganisms are being cultured.

Over long incubation periods on culture media formulated for microbial growth, mammalian cells do not survive, and their ATP is degraded. However, if incubation is short mammalian ATP can still be detected by bioluminescence.

We have previously demonstrated that the selective lysis of mammalian cells, prior to sample filtration, reduces the background signal caused by intracellular mammalian ATP. The composition of the differential lysis buffer used for this purpose had to be modified to comply with changes to European Chemicals Agency (ECHA) guidelines.

The effectiveness of the modified lysis buffer and microbial detection with Milliflex® Rapid System 2.0 were evaluated using mammalian cell culture samples artificially contaminated with various microorganisms (Gram-/Gram+ bacteria, yeast and molds).

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Material and Method

Microbial strains and incubation

Microorganisms listed in **Table 1** were resuspended in NaCl Peptone Water at a concentration of 1 x 10^3 cfu/mL.

Microorganism	Reference Strain	Incubation	Time to Results (h)
Bacillus spizizenii	WDCM 00003	30–35 °C aerobic	8
Staphylococcus aureus	WDCM 00032	30–35 °C aerobic	16
Pseudomonas paraeruginosa	WDCM 00026	30–35 °C aerobic	16
Clostridium sporogenes	NCTC 12935	30–35 °C anaerobic	20
Candida albicans	WDCM 00054	20–25 °C aerobic	24
Aspergillus brasiliensis	NCPF 2275	20–25 °C aerobic	48
Cutibacterium acnes	DSM 1897	30–35 °C anaerobic	72

Table 1: Microorganism incubation conditions

Cell culture

Chinese hamster ovary CHO-S cells, derived from CHO-K1 cells and adapted to serum-free suspension culture, were grown in shaker flasks in supplemented serum-free CD-CHO medium up to a concentration of 1 x 10^7 cells/mL.

Mammalian cell lysis and ATP hydrolysis

Under aseptic conditions, 1 mL of CHO-S cells at a concentration of 1 x 10^7 cells/mL were mixed with 9 mL of the modified lysis buffer*, 5U of Apyrase (**Cat. No. A6132**) and around 50 cfu of each microorganism strain. The mix was gently vortexed for 4 seconds and incubated for 15 minutes at room temperature.

*For more details about the lysis buffer, please contact us.

Milliflex Oasis® membrane filtration

Under aseptic conditions, a Milliflex Oasis® membrane was wetted by filtering 10 mL of Fluid D. 20 mL of NaCl peptone buffer were then poured into the filtration funnel, followed by the lysed and artificially contaminated cell sample. The total sample volume was adjusted in the funnel to 100 mL using NaCl peptone buffer and subsequently filtered using a Milliflex Oasis® pump. The membrane was then rinsed 3x with 100 mL of Fluid D, then with 100 mL of NaCl peptone buffer, and then transferred onto a RSTM cassette, which contains a specific microbial culture medium developed for rapid testing of various product types. Subsequent incubation was performed as stated in **Table 1** above.

Milliflex® Rapid detection

Following the Milliflex® protocol, the membrane was removed from the RSTM cassette and left to air-dry in a laminar flow cabinet. The ATP detection reagents were then sprayed on the membrane using the Milliflex® Rapid System 2.0 AutoSpray Station. The membrane was then read with the Milliflex® Rapid System 2.0 Detection Tower, which automatically determines the number of cfu on the membrane.

Results

The results for three test conditions were determined and analyzed (A, B and C in **Table 2**). All conditions involved the addition of one of the microorganism strains, membrane filtration and Milliflex® rapid detection. For condition C, mammalian cells were added, and the lysis step performed to remove their ATP. For condition B, the mammalian cells were also added but no lysis performed, retaining their ATP. For condition A, neither mammalian cells were added nor lysis performed, leaving only the ATP of the microorganisms to be detected.

Test Condition	Micro organisms	Mammalian cells	Lysis + ATP removal	Membrane filtration	Milliflex® Rapid detection
A (control)	+	-	-	+	+
B (no lysis)	+	+	-	+	+
C	+	+	+	+	+

Table 2: Test condition summary

Per condition and per microorganism strain, five replicate RSTM cassettes were inoculated and incubated. Grown microcolonies were detected by ATP bioluminescence and automatically counted using the Milliflex® Rapid System 2.0 Detection Tower (result example for *B. spizizenii* in **Figure 1**, top panel).

For each microbial strain, the averages of the replicates under test conditions B and C were determined as percentages of the replicate average under the control condition A, which was set to 100% (**Figure 1**, bottom panel).

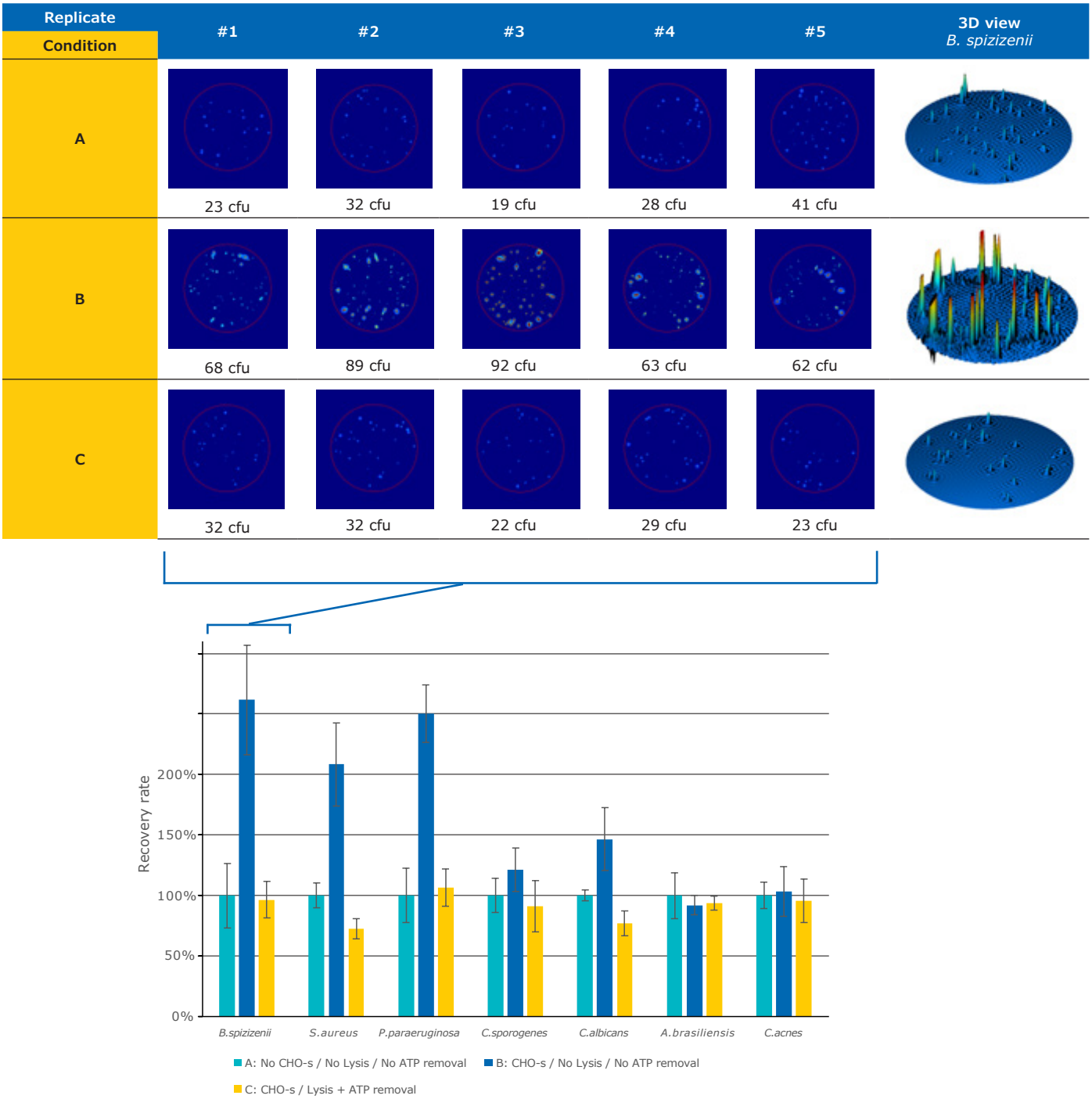

Figure 1: Detection images and counts of microcolonies using the Milliflex® Rapid System 2.0 detection protocol for bioburden testing under conditions A, B and C. Top panel: 2D images of the 5 replicate RSTM cassettes and corresponding cfu counts automatically generated by image processing (example of *B. spizizenii*), on the right representative 3D view of signal intensity for one replicate as read by the Milliflex® Rapid System 2.0 Detection Tower. Bottom panel: Recovery rate averages expressed as percentages of the test condition A averages, determined by the Milliflex® Rapid System 2.0 Detection Tower. The bar in each column indicates the standard deviation (n=5).

Comparison of conditions A and B (**Figure 1**) shows that the Milliflex® Rapid System 2.0 Detection Tower detected and counted more ATP-containing spots on the membrane as cfu when unlysed mammalian cells were present in the sample. The difference was particularly pronounced for fast-growing strains with short incubation periods. However, sample treatment with the new selective mammalian cell lysis buffer followed by ATP hydrolysis (**Figure 1**, condition C) prior to filtration strongly decreased the non-specific signals in the samples of fast-growers, with no impact on microbial ATP detection. The microorganism recovery rate after sample treatment ranged between 72% and 106%. Therefore, the mammalian cell lysis and ATP removal protocol did not interfere with microorganism growth and detection.

Discussion

Quantifying viable contaminant microorganisms with ATP bioluminescence detection platforms still represents a challenge when monitoring biomanufacturing processes designed to produce mammalian cell-based therapeutics. As mammalian ATP also reacts with the detection reagents, the cfu counts are sometimes highly overestimated if the samples are not pre-treated to remove this mammalian ATP. This is especially the case for fast growing microorganisms with short incubation times such as *P. paraeruginosa* and *S. aureus*, and generally for growth conditions where mammalian ATP is not fully hydrolyzed by the time of microcolony detection and counting.

Another known benefit of lysing the mammalian cells, not subject of this study, is that this improves Milliflex® membrane filtration: the Milliflex® membrane is less likely to get clogged by mammalian cells, which can be an issue when the mammalian cell concentration in the sample is high.

This study demonstrates that, in a lysis step that takes 15 minutes to complete, the modified lysis buffer can significantly reduce false-positive signals caused by viable mammalian cells in samples and thus enable reliable rapid detection and enumeration of microbial contaminants using the Milliflex® Rapid System 2.0.

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