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Preparation, Separation,
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Application Note

Rapid fluorescence-based detection of microbial contamination in ethanol and gel-based disinfectant

Matrix compatibility of the Milliflex® Quantum system when using MCE and PVDF membrane filters after neutralizing antimicrobial activity

In the current pandemic, frequent disinfection of surfaces, objects and hands are key measures in public healthcare. As a result, manufacturers face an urgent and growing demand for ethanol and gel-based disinfectants. These samples must undergo QC controls to ensure that the bioburden is below an acceptable level. Membrane filtration is the most widely used method for release testing. If used in conjunction with rapid bioburden testing methods, it allows final products to be released earlier to the market than when using compendial methods.[1,2]

The fluorescence-based Milliflex® Quantum technology offers a convenient and sensitive method for the quantitative rapid detection of contaminants in filterable samples. It can detect microbial contamination up to three times faster than traditional plate-based monitoring methods. The rapid microbial method is based on a universal enzymatic fluorescent staining of viable and culturable microorganisms that allows microcolonies to be detected before they become visible to the naked eye. The fluorescent staining procedure is non-destructive, making it possible to identify microorganisms following a positive result.

The aim of the study was to demonstrate the Milliflex® Quantum system's capability to detect the bioburden in ethanol and gel-based disinfectants using Milliflex Oasis® MCE and PVDF membrane filters. Furthermore, the study describes an effective way to neutralize the bactericidal activity of the two alcohol-containing matrices.

Material

Bacillus subtilis ATCC®[3] 6633™ was selected as anaerobe spore-forming strain and *Staphylococcus aureus* ATCC® 6538™ as mandatory test strain named in EN 13727-2012 which describes the suspension test for the evaluation of bactericidal activity of chemical disinfectants.

Table 1: Filtration matrices and rinsing solution

	Product	Cat. No
Matrices	Ethanol absolute EMPLURA®	**8187601000** Ingredient: close to 100% ethanol
	Gel-based disinfectant	Provided by a certified pharmacy Ingredients: isopropanol 70%, hydroxyethyl cellulose 300
Rinsing solution	Buffered NaCl-peptone solution	**1.46368.0006**

Table 2: Hardware and consumables

Product	Cat. No.
Milliflex Oasis® filtration pump	**MMSYSTMM1**
Milliflex Oasis® filtration unit, volume 100 mL, gridded white mixed cellulose ester (MCE) filter, pore size 0.45 µm	**MMHAWG125**
Milliflex Oasis® Funnel, plain white filter, PVDF membrane, pore size 0.45 µm	**MMHVWP124**
Milliflex Oasis® tryptic soy media plates for detection of aerobic and anaerobic flora	**MMSMCTS48**
Milliflex® Liquid Media Cassette	**MXLMC0120**
Milliflex® Quantum Reagent Kit	**MXQREAG48**
Milliflex® Quantum Rapid Detection System	**MMQUANK01**

Method

The first focus of this study was to compare the microbial growth promotion and detection performance for the two sample types when using MCE (mixed cellulose ester) and PVDF (polyvinylidene difluoride) membrane filters for filtration. The second focus was to assess the method's efficiency of neutralizing bactericidal activity in the ethanol and isopropanol matrices.

The method used in this study was based on a pharmaceutical customer's release-testing procedure of absolute ethanol. The plates were incubated for either 19 or 24 hours, depending on the target microorganism. The optimal incubation time for rapid contaminant detection with Milliflex® Quantum was not determined as the study's aim was to demonstrate compatibility, not performance levels.

The tested matrices were absolute ethanol and a gel-based disinfectant containing isopropanol as active ingredient (**Table 1**). The growth-inhibiting properties of both alcohol-containing samples were neutralized with NaCl-peptone as described below.

Membrane filtration method: Neutralization of active ingredients

For both samples, NaCl-peptone was chosen as neutralizer. Prior to filtration, **10 mL of sample** (ethanol for *part I* section and the gel-based disinfectant for *part II* section) were mixed with **90 mL of NaCl-peptone** to create the so-called **matrix solution**. For each filtration, the membrane filter was pre-wet with 50 mL of NaCl-peptone and the matrix solution subsequently filtered. Then, the membrane filter was rinsed three times: the first two rinsing steps were performed with 100 mL of NaCl-peptone and the final one with 100 mL of NaCl-peptone spiked with 10 to 100 colony forming units (CFU) of the respective target microorganism.

Ten replicates were performed per matrix and per membrane filter type (five replicates for rapid detection using the Milliflex® Quantum method and five for traditional counting as a control). Two target microorganisms, *Bacillus subtilis* and *Staphylococcus aureus*, were tested on ethanol, and one target microorganism, *Staphylococcus aureus*, on the gel-based disinfectant.

For traditional readings, as well as for Milliflex® Quantum detection of colonies, the samples were incubated at 32.5 °C ± 2.5 °C on Milliflex Oasis® TSA cassettes for:

- 19 hours for *Bacillus subtilis*
- 24 hours for *Staphylococcus aureus*

To calculate the inoculation level, controls were spread on TSA 90 mm plates without any prior treatment. 5 plates were spread for *B. subtilis* and 5 plates for *S. aureus*.

Table 3: Method overview

Bacterial stain and ATCC® number	Matrix: Ethanol*	Matrix: gel-based disinfectant*	Incubation temperature	Incubation time	Media	Traditional detection	Rapid detection
Bacillus subtilis ATCC® 6633™ - WDCM 00003	Tested	Not tested	32.5 °C +/- 2.5 °C	19 h	TSA	5 MCE and 5 PVDF	5 MCE and 5 PVDF
Staphylococcus aureus ATCC® 6538™ - WDCM 00193	Tested	Tested	32.5 °C +/- 2.5 °C	24 h	TSA	5 MCE and 5 PVDF	5 MCE and 5 PVDF

* both matrixes were neutralized with NaCl-Peptone

Milliflex® Quantum method: Recovery rates

After incubation, the microcolonies were detected via the Milliflex® Quantum fluorescence-based method. The fluorogenic substrate used is a non-fluorescent viability marker that is cleaved by non-specific ubiquitous intracellular enzymes, resulting in a fluorescent signal.

To evaluate the effect of ethanol on the Milliflex® Quantum system's performance (method reliability), half of the tested membrane filters were stained with the Milliflex® Quantum system's reagent and the results compared with those on unstained membrane filters, with a focus on colony numbers and morphology. The following formula was used to calculate the recovery rate:

$$\text{Recovery rate (\%)} = \frac{\text{Average of counts [stained or unstained membrane filters]}}{\text{Average of traditional method counts [spread plate technique]}}$$

Acceptance criterion: All recovery rates must be above 70% as recommended in the "Alternative Methods for Control of Microbiological Quality" chapters of the pharmacopeias.

Part I: Matrix ethanol

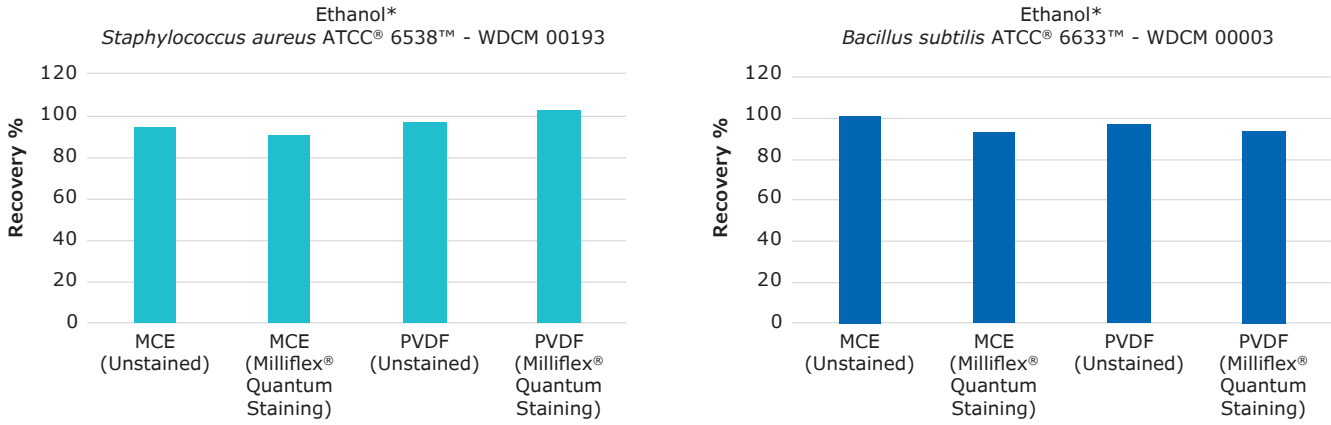

Figure 1: Recovery rates for detecting contaminants in ethanol matrices using the Milliflex Oasis® and Milliflex® Quantum systems

The ethanol matrix was neutralized with a NaCl-peptone solution, then filtrated through Milliflex Oasis® filtration devices. MCE and PVDF membrane filters were tested. After a 24-hour incubation for *Staphylococcus aureus* and a 19-hour incubation for *Bacillus subtilis*, five membrane filters each were analyzed with Milliflex® Quantum to detect fluorescent microcolonies (Milliflex® Quantum Staining). As a control, five other membrane filters were evaluated with the naked eye for traditional counting of the colonies (Unstained).

* Neutralized with NaCl-peptone.

The results show recovery rates of 90% and above for both membrane types and both target microorganisms. Hence the ethanol's antimicrobial activity was successfully neutralized by the addition of NaCl-peptone over the 3 rinsing steps, proving that the Milliflex® Quantum staining reaction works well when ethanol is sufficiently eliminated.

All results were found to be above the acceptance threshold (**Figure 1**), with good Milliflex® Quantum recovery rates for *B. subtilis* (91% on MCE and 103% on PVDF membrane filters) and *S. aureus* (92% on MCE and 94% on PVDF membrane filters). The colony morphology was found to be as expected (**Figure 2**) and similar to the controls. These results confirm that the NaCl-peptone solution, used over 3 rinsing steps, effectively neutralizes the antimicrobial activity of the ethanol matrix. No significant differences were observed between results generated on MCE and PVDF membrane filters.

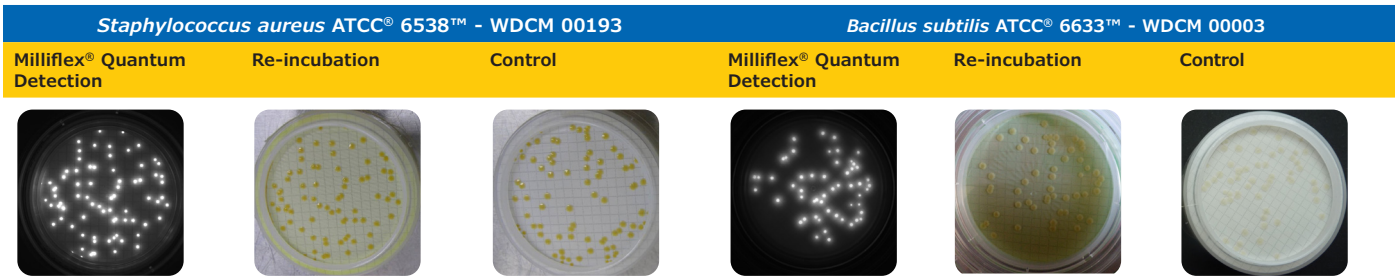

Figure 2: *Staphylococcus aureus* and *Bacillus subtilis* observation by Milliflex® Quantum and after re-incubation.

The NaCl-peptone neutralized ethanol matrix was inoculated with either *S. aureus* or *B. subtilis*, then filtrated through MCE membrane filters on Milliflex Oasis® filtration devices. *S. aureus* colonies were observed with the Milliflex® Quantum system after a 24-hour incubation (Milliflex® Quantum detection). Then the membrane filters were re-incubated on a fresh Milliflex® cassette for subsequent identification (Re-incubation). After a 19-hour incubation, the *B. subtilis* colonies were detected by the Milliflex® Quantum system (Milliflex® Quantum Detection). Then, membrane filters were re-incubated on a fresh Milliflex cassette for subsequent identification (Re-incubation). For each strain a traditional culture on Milliflex Oasis® TSA plates was performed.

Part II: Matrix gel-based disinfectant

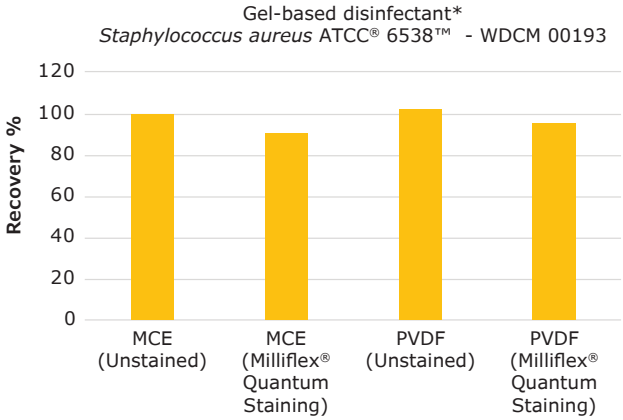

Figure 3: Recovery rates for detecting contaminants in the gel-based disinfectant matrices using the Milliflex Oasis® and Milliflex® Quantum systems

MCE and PVDF membrane filters were tested. After a 24-hour incubation, five membrane filters were analyzed with Milliflex® Quantum to detect fluorescent microcolonies (Milliflex® Quantum Staining). As a control, five other unstained membrane filters were evaluated with the naked eye for a traditional count of the colonies (Unstained).

* Neutralized with NaCl-peptone.

All results were found to be well above the acceptance threshold (**Figure 3**), with good Milliflex® Quantum recovery rates for the target microorganism *S. aureus* (91% on MCE and 96% on PVDF membrane filters). The colony morphology was found to be as expected (**Figure 2**, *S. aureus*) and similar to controls. These results confirm that the NaCl-peptone solution, used over 3 rinsing steps, effectively neutralizes the antimicrobial activity of the isopropanol matrix. No significant differences were observed between the results generated on MCE and PVDF membrane filters.

Conclusion

The Milliflex® Quantum system was found to be suitable for efficiently detecting microbial contamination in absolute ethanol and gel-based, isopropanol-containing disinfectant when applying a procedure that neutralizes the alcohol-related antimicrobial activity.

The Milliflex® Quantum rapid detection system reduces the time needed to detect microbial contamination by up to two-thirds compared to the traditional method. The fluorescent staining procedure is non-destructive, allowing the identification of the contaminant following a positive result. Combined with the new Milliflex Oasis® platform, the Milliflex® Quantum system is a powerful tool for manufacturers wanting to accelerate product release and decrease storage times.

Literature

1. Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area – Test method and requirements (phase 2, step 1); English version EN 13727:2012+A2:2015, English translation of DIN EN 13727:2015-12
2. European Pharmacopoea 5.1.6 - Alternative Methods for Control of Microbiological Quality
3. ATCC®: American Type Culture Collection (ATCC) is a registered trademark of a nonprofit company

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