MILLIPORE

Specific Detection of *Pseudomonas aeruginosa* in Water

Using the Milliflex® Rapid Microbiology Detection and Enumeration System

Introduction

Water is a critical raw material used in the manufacture of pharmaceutical and cosmetic products. Water testing represents approximately 70% of the total microbial quality control tests performed using the membrane filtration (MF) method. To ensure final product quality, manufacturers regularly monitor for bioburden throughout the manufacturing process.

Pseudomonas aeruginosa is one contaminant that is often found in water. It is a gram-negative bacterium that is noted for its environmental versatility. It has the exceptional ability to colonize ecological niches where nutrients are limited, such as in purified water and water for injection (WFI). *P. aeruginosa* is a source of endotoxin and can cause disease in susceptible individuals. As a result, water used to manufacture pharmaceutical products as well as a number of cosmetic products must meet the various regulatory requirements that are in place to ensure the absence of *P. aeruginosa*.

Pharmaceutical Water Testing

The United States Pharmacopeia (USP) states in Chapter <1231> "Water for Pharmaceutical Purposes" that an action level event for the monitoring of a water system can be reached by an individual or repeated recovery of specific objectionable microorganisms.

Cosmetics Water Testing

Within the European Union, *P. aeruginosa* must not be detectable in 0.5 g or 0.5 mL of cosmetic products to be used for the eye area, mucous membranes or for cosmetic products specifically

intended for children under 3 years. In other cosmetics, *P. aeruginosa* must not be detectable in 0.1 g or 0.1 mL (European Commission,1999, Notes of guidance for testing of cosmetic ingredients for their safety evaluation, Guidelines Cosmetic Products, Vol. 3. pp. 57–58).

Specific Detection of *Pseudomonas aeruginosa* in Hours, Not Days

The Milliflex Rapid system is a proven automated solution for the rapid detection and enumeration of total viable count (TVC) in purified water and WFI. Based on membrane filtration and image analysis together with an adenosine triphosphate (ATP) bioluminescence reagent, the Milliflex Rapid System delivers TVC test results faster than traditional detection methods. Millipore has developed a hybridization assay that will enable the Milliflex Rapid system to specifically detect and enumerate *P. aeruginosa*. The hybridization assay is performed with a peroxidase-conjugated DNA-oligonucleotide probe targeted to a specific RNA-sequence of *P. aeruginosa*. Applying luminol and peroxide substrates to the membrane filtration sample generates light that is detected by the Milliflex Rapid system. The Milliflex Rapid image analysis software measures the light generated by the reaction and transforms the data to familiar colony forming units (CFUs).

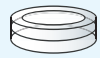
Figure 1. *P. aeruginosa* Specific Detection and Enumeration Protocol

Step 1: Filtration. Sample filtration on Milliflex membrane.

Step 2: Incubation. Incubate on media to stimulate organism growth.

Step 3: Permeabilization. Permeabilization of cells on the membrane.

Step 4: Fixation. Fixation of organisms on the membrane.



Step 5: Pre-hybridization. Pre-hybridization to prevent non-specific binding.

Step 6: Hybridization.

Step 7: Washing. Wash away extra probes

Step 8: Spraying. Spray detection reagents.

Step 9: Imaging. Place sample on the Milliflex Rapid System to detect and enumerate *P. aeruginosa* in CFUs.

Materials

Hardware

- Milliflex Rapid Microbiology Detection and Enumeration System
- Milliflex Rapid AutoSpray Station
- Milliflex PLUS vacuum pump
- 32.5 °C ± 2.5 °C incubator
- 25 °C ± 2.5 °C incubator
- 50 °C ± 2.5 °C incubator
- 50 °C ± 2.5 °C shaker (Polymax 1040 Heidolph)
- Laminar flow hood

Consumables/Media/Reagents

- Milliflex Rapid and Milliflex funnels with 0.45 µm polyvinylidene fluoride (PVDF) membrane (Cat. nos. RMHV MFX 24 and MXHV WP1 24)
- Milliflex-100 funnels with 0.45 µm mixed cellulose esters (MCE) membrane (Cat. no. MXHA WG1 24)
- Prefilled TSA Milliflex cassettes (Cat. no. MXSM CTS 48)
- Prefilled R2A Milliflex cassettes (Cat. no. MXSM CRA 48)
- Sterile 0.9% NaCl water
- Milliflex Rapid reagent kit (Cat. no. MXRP BLR ST)
- Milliflex Rapid *Pseudomonas aeruginosa* detection kit (Cat. nos. MXRP SEU D0, MXRP ROB PA, MXRE XPE ND)

Strains of Common Waterborne Microorganisms

- *Pseudomonas aeruginosa* (ATCC® 9027)
- *Serratia marcescens* (ATCC 14756)
- *Klebsiella pneumoniae* (ATCC 13883)
- *Escherichia coli* (ATCC 25922)
- *Burkholderia cepacia* (ATCC 25416)
- *Proteus mirabilis* (ATCC 29906)
- *Ralstonia pickettii* natural isolate
- *Bacillus cereus* (ATCC 11778)

Methods

Total Viable Count: Determination of Incubation Time

This procedure was used to determine the minimum incubation time necessary to detect and enumerate microorganisms with the Milliflex Rapid System.

1. Pour 50 mL of sterile 0.9% NaCl solution into a RMHV MFX 24 Milliflex Rapid funnel.
2. Spike the appropriate dilution of each microorganism into the funnel.
3. Add 50 mL of sterile 0.9% NaCl solution into the funnel to homogenize the content.
4. Filter and transfer the membrane onto a prefilled TSA Milliflex cassette.
5. Incubate at 32.5 °C ± 2.5 °C.
6. Once incubation is complete separate the membrane from the cassette and let the membrane dry.
7. Spray the Milliflex Rapid reagents (ATP releasing agent and bioluminescence reagent) using the Milliflex Rapid AutoSpray Station.
8. Read the sample with the Milliflex Rapid system.
9. Test different incubation times until the optimal time is determined for each microorganism.

Steps 1 through 4, 6 and 7 are performed inside a laminar flow hood.

The same filtration and incubation protocol, as described above, was used with MXHA WG1 24 Milliflex funnels (control method) to determine the incubation time necessary for the detection of each microorganism using the traditional microbiology method.

The filtrations were performed in replicates of five for both the traditional microbiology method and Milliflex Rapid System for each microorganism.

Specific Detection and Enumeration of *P. aeruginosa*

This procedure was used to determine the minimum incubation time necessary to detect and enumerate *P. aeruginosa* with the Milliflex Rapid System

1. Pour 50 mL of sterile 0.9% NaCl solution into a MXHV WP1 24 Milliflex funnel.
2. Spike the appropriate dilution of each microorganism into the funnel (10–100 CFUs).
3. Add 50 mL of sterile 0.9% NaCl solution into the funnel to homogenize the content.
4. Filter and transfer the membrane onto a prefilled TSA Milliflex cassette.
5. Incubate at 32.5 °C ± 2.5 °C for the appropriate time.
6. Once incubation is complete, separate the membrane from the cassette and let the membrane dry.
7. Follow the Milliflex Rapid *Pseudomonas aeruginosa* detection procedure (see User Guide PF10305).
8. Spray the specific detection reagents using the Milliflex Rapid AutoSpray Station.
9. Read the sample with the Milliflex Rapid Detection and Enumeration System.

Steps 1 through 4, 6 and 8 were performed inside a laminar flow hood.

Combination of Total Viable Count and Specific Detection of *P. aeruginosa*

This procedure was used to obtain both total viable count and specific detection and enumeration of *P. aeruginosa* using the same membrane sample.

1. Pour 50 mL of sterile 0.9% NaCl solution into a MXHV WP1 24 Milliflex funnel.
2. Spike the appropriate dilution of each microorganism into the funnel (10–100 CFUs).
3. Add 50 mL of sterile 0.9% NaCl solution into the funnel to homogenize the content.
4. Filter and transfer the membrane onto a pre-filled TSA Milliflex cassette.
5. Incubate at 32.5 °C ± 2.5 °C for the appropriate time.
6. Once incubation is complete, separate the membrane from the cassette and let the membrane dry.
7. Spray the ATP releasing and bioluminescence reagents using the Milliflex Rapid AutoSpray Station.
8. Read the sample with the Milliflex Rapid Detection and Enumeration System.
9. Place membrane on a pad soaked with 0.25% H_2O_2 for 10 minutes.
10. Follow the Milliflex Rapid *Pseudomonas aeruginosa* detection procedure starting from fixation step number two (see User Guide PF10305).
11. Spray the specific detection reagents using the Milliflex Rapid AutoSpray Station
12. Read the sample with the Milliflex Rapid Detection and Enumeration System.

Steps 1 through 4, 6, 7, 11 and 12 were performed inside a laminar flow hood.

Results

Total Viable Count: Determination of Incubation Time

Table 1 provides the minimum incubation time necessary for detectable growth, by organism, for the traditional microbiology method and the Milliflex Rapid System.

Recovery between the Milliflex Rapid system and the traditional microbiology method in these experiments was between 70% and 130%, which demonstrates the equivalence of the two methods for the enumeration of viable microorganisms as stated in the PDA Technical Report 33 and USP Proposed Chapter 1223.

Specific Detection of *P. aeruginosa*

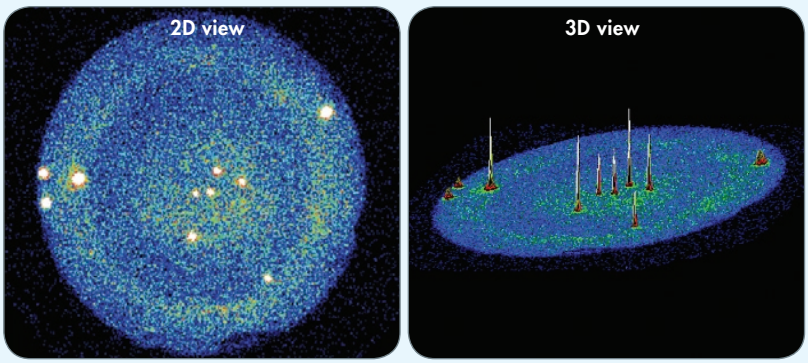
For the Milliflex Rapid method, a pure culture of *P. aeruginosa* ATCC 9027 was filtered through a Milliflex funnel and incubated on TSA for 6.5 hours. For the control method, a pure culture of *P. aeruginosa* ATCC 9027 was filtered through a Milliflex funnel and incubated on R2A for 24 hours. Results are presented in Figure 2.

Using the specific detection procedure described above, *P. aeruginosa* was detected and enumerated in 8.5 hours in a water sample. The specificity of the method has been assessed against numerous microorganisms and only *P. aeruginosa* was detected in this panel of contaminants (refer to the Milliflex Rapid *Pseudomonas aeruginosa* Specific Detection Assay data sheet, DS1033EN00). The limit of the sensitivity is 1 CFU.

Table 1. Minimum Incubation Time and Percent Recovery using the Milliflex Rapid System

	Traditional Microbiology	Rapid Microbiology		
Microorganisms	Count (CFUs)	Minimum Incubation Time	Count (CFUs)	% Recovery
Pseudomonas aeruginosa	48	9 h	40	83 %
Serratia marcescens	53	7 h	40	75 %
Klebsiella pneumoniae	11	8 h	8	73 %
Escherichia coli	21	6 h 30	15	71 %
Burkholderia cepacia	44	15 h	33	75 %
Proteus mirabilis	47	8 h	38	81 %
Ralstonia pickettii	21	15 h	15	71 %
Bacillus cereus	14	8 h	16	114 %

Figure 2. Specific Detection and Enumeration of *P. aeruginosa* using the Milliflex Rapid System



Milliflex Rapid Specific Detection Count
[TSA, 32.5 °C ± 2.5 °C]:
10 CFUs

Traditional Microbiology Count
[R2A, 25 °C ± 2.5 °C]:
11 CFUs

Recovery:
91%

Combining TVC and Specific Detection on a Single Membrane

The objective of this experiment was to first obtain the TVC in CFUs using the total viable count assay, followed by the specific detection assay for *P. aeruginosa*. After performing the TVC analysis, the results were stored on the Milliflex Rapid system and the same membrane was then treated following the specific detection procedure. The TVC and the specific detection count data were then analyzed. The results are presented in Figure 3.

Figure 3 provides results for both TVC and specific detection of *P. aeruginosa* using the same membrane. The images above show that the position of each colony forming unit is identical when using the TVC and specific detection assay. One hundred percent of the CFUs were detected in each assay.

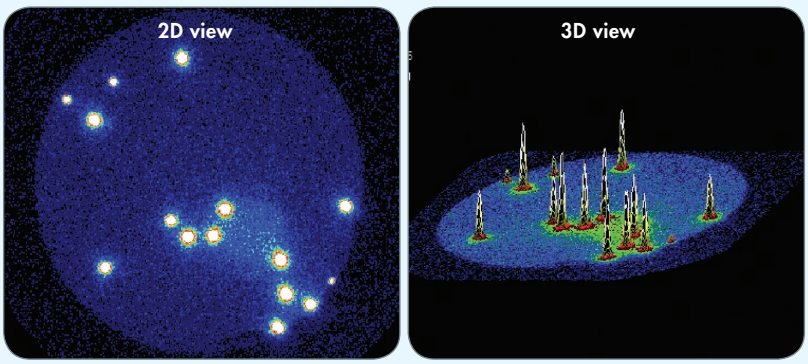
Specific Detection of *P. aeruginosa* in a Mixed Population after TVC on the Same Membrane

A mixed microbial population composed of *P. aeruginosa, B. cepacia and E. coli* were spiked and analyzed with the procedure described in "Combination of Total Viable Count and Specific Detection of *P. aeruginosa*." Results are presented in Figure 4.

After 9 hours growth at 35 °C, 24 CFUs were detected after the TVC procedure and 7 CFUs were detected using the *P. aeruginosa* specific detection procedure.

Figure 3. Specific Detection of *P. aeruginosa* after TVC on the Same Membrane using a Pure Culture of *P. aeruginosa* ATCC 9027 [TSA, 32.5 °C ± 2.5 °C]

Total Viable Count (TVC)



Milliflex Rapid Microbiology TVC:
15 CFU

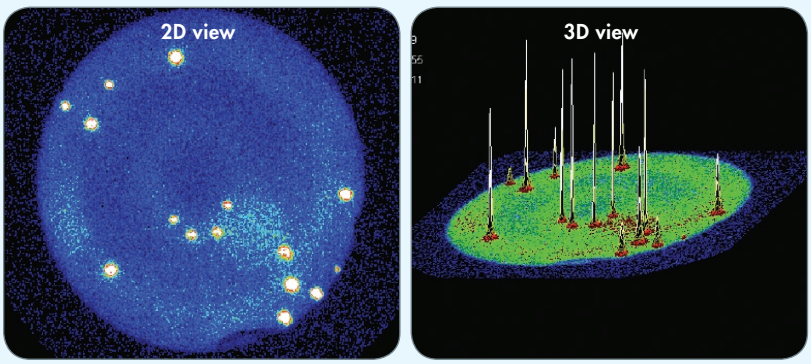
Milliflex Rapid *P. aeruginosa* Specific Detection Count:
15 CFU

Recovery:
100%

Incubation Time:
9 h

Overall Time to Result:
11 h 30

P. aeruginosa



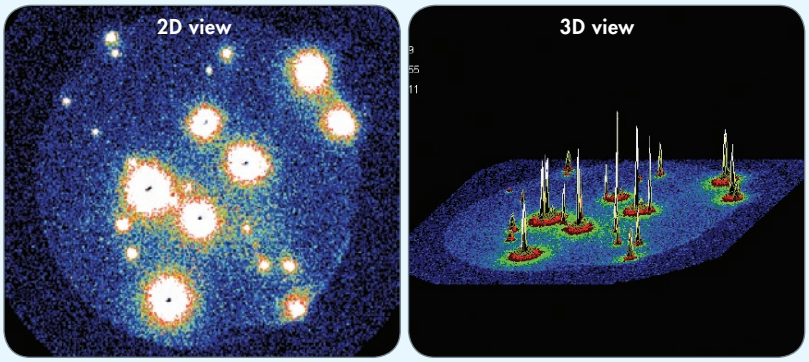
Conclusion

The Milliflex Rapid detection system allows detection and enumeration of viable microorganisms while significantly decreasing the time to result. The system enables the user to take advantage of two forms of information: Total viable count as well as the specific detection and enumeration of *P. aeruginosa*.

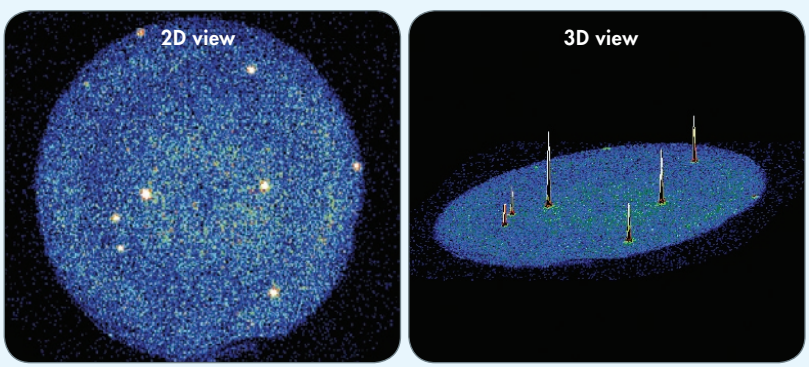
The methods described in this paper enable pharmaceutical and cosmetic product manufacturers to quickly detect and enumerate microbial contamination in their water systems. This provides manufacturers with an opportunity to implement corrective actions in their water monitoring programs much earlier than traditional methods would allow.

Figure 4. Specific Detection of *P. aeruginosa* after TVC on the Same Membrane using a Mixed Population of *P. aeruginosa* ATCC 9027, *B. cepacia* ATCC 25416 and *E. coli* ATCC 25922 [TSA, 32.5 °C ± 2.5 °C]

Total Viable Count (TVC)



P. aeruginosa



Milliflex Rapid Microbiology TVC:
24 CFUs

Milliflex Rapid *P. aeruginosa* Specific Detection Count:
7 CFUs

Percentage of *P. aeruginosa* Contaminants in the Mix:
29%

Incubation Time:
9 h

Overall Time to Result:
11 h 30

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