Application Note

Milliflex® Quantum – Detection of microbial contaminants in water samples

Water is a key raw material utilized in the manufacturing of products within the pharmaceutical, biopharmaceutical and healthcare industries. Within each industry, regulatory requirements exist for microbial contamination in different levels of water purity. The microorganisms found in these water systems are mainly stressed, slow-growing strains characterized by long incubation times (5 to 7 days) before growth can be detected using traditional microbiology methods. The time required before contamination can be detected in water can cause delays in product release, and extend the storage time of products. By using rapid detection methods, manufacturers are able to address contamination events sooner, avoid line shutdowns, release product to the market faster, and reduce warehousing costs.

The Milliflex® Quantum system is a convenient platform for the rapid detection of microbial contamination in filterable samples. The system is based on a non-destructive fluorescence labelling of viable and culturable microorganisms collected on a Milliflex® mixed cellulose esters (MCE) membrane. After filtration and incubation, microorganisms retained on the membrane are labelled by a fluorescent marker. The reaction consists of an enzymatic cleavage by active microorganism metabolism of a non-fluorescent substrate. Once cleaved inside the cell, the free fluorochrome is liberated into the microorganism cytoplasm. As fluorochrome accumulates inside the cells, the signal is naturally amplified. The cells are then exposed to the excitation wavelength of the fluorescent dye in the Milliflex® Quantum reader so that the colonies can be counted visually. Detected micro-colonies can be recovered by membrane re-incubation after the staining step allowing for further identification using existing ID methodologies.

Materials:
Milliflex® Funnels, Mixed Cellulose Esters (MCE) 0.45µm (MXHAWG124)
Milliflex® R2A Cassettes (MXSMCRA48)
Milliflex® Liquid Cassette (MXLMC0120)
Milliflex® Quantum Reagent Kit (MXQTV0KT1)

Equipment:
Milliflex® PLUS Pump (MXPPLUS03)
Milliflex® Quantum System (MXQUANK01): reader, camera, stand, membrane transfer tool, removal rack, and Quantum Spot Counter software
Incubator, 32.5 +/- 2.5°C

Method Overview:

Various water samples (lab water and purified water) were tested in order to assess the ability of the Milliflex® Quantum System to detect microbial contaminants quicker and to evaluate the reduction of time-to-final result in comparison with the compendial method.

The water sample was filtered through a Milliflex® 0.45 µm MCE membrane using the Milliflex® PLUS pump system. The membrane was placed onto a prefilled R2A Milliflex® agar media cassette and incubated at 32.5 +/- 2.5 °C in aerobic conditions for different incubation times.

After incubation, the membrane was disconnected from the media cassette using the Removal Rack and placed onto a Milliflex® liquid cassette pre-saturated with 2mL of the Quantum staining solution. The stained membrane was incubated at 32.5 +/- 2.5 °C for 30 min then read using the Milliflex® Quantum reader. The stained membrane was re-incubated on a new R2A agar media cassette to determine microbial recovery after staining. Refer to Figure 1 for an overview of the Milliflex® Quantum protocol.

The compendial method was performed in parallel and incubated for up to 7 days for final visual counting of colony forming units (CFU).

Figure 1. Milliflex® Quantum Protocol Overview

For each incubation time point using the Milliflex® Quantum system, the average fluorescence count and average Colony Forming Unit (CFU) count obtained after re-incubation of stained membranes were compared to the average count of the Milliflex® compendial method. The fluorescence recovery and re-incubation recovery parameters were calculated as follows:

$$\text{Fluorescence recovery}\,(\%) = \frac{\text{Fluorescence count}}{\text{Milliflex® compendial method count}} \times 100$$

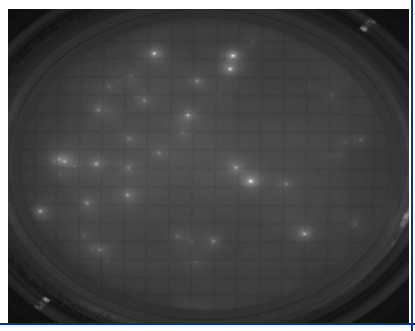
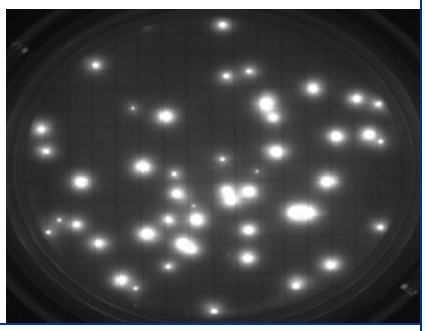
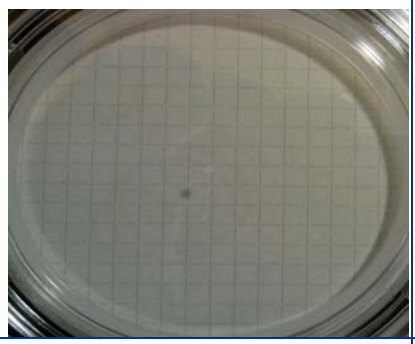
$$\text{Reincubation recovery}\,(\%) = \frac{\text{CFU count after reincubation}}{\text{Milliflex® compendial method count}} \times 100$$

Acceptance criteria for each of these parameters are set to 70% or greater, which is the lower limit of acceptance to replace the conventional method by an alternative one (General Information Chapters United States Pharmacopeia <1223> and European Pharmacopeia 5.1.6).

Results - Lab Water:

Lab water samples were incubated for 24 and 30 hours to determine whether or not contamination could be detected quicker using the rapid method as compared to the traditional method. After 24hr incubation, micro-colonies were detected using the Milliflex® Quantum system whereas no visible colonies were detected using the traditional method. The traditional method required a minimum of 30hr incubation in order to visually detect macro-colonies, but they were extremely small and only a third of the count as compared to the rapid method. Refer to Table 1 for results.

Table 1: Detection of contamination in lab water after different incubation times; results are an average of three membrane replicates.

Incubation times	24 hr	30 hr
Milliflex® Quantum camera		
Milliflex® Quantum fluorescent counts	~ 43	56
Compendial method		
Compendial method counts	0	~ 16

Results - Purified Water:

Purified water samples, each from a different process of a pharmaceutical plant, were incubated for 24 and 30 hours to evaluate the reduction of time-to-final result in comparison with the compendial method. Water sample 1 required 30hr incubation and water sample 2 required 24hr incubation in order to achieve > 70% recovery as compared to the compendial method. After re-incubation of the stained membranes, >70% recovery was achieved for both types of purified water samples as compared to the compendial method. Refer to Table 2 for results and Table 3 for a comparison of colonies after staining and re-incubation.

Table 2: Recovery of contamination in purified water after different incubation times; results are an average of 5 membrane replicates.

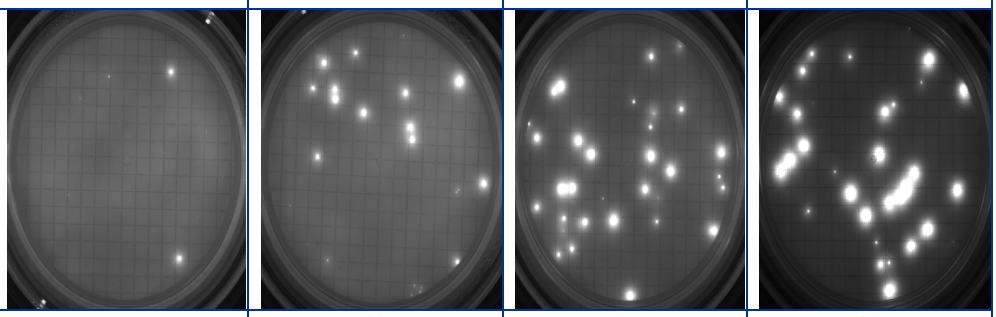
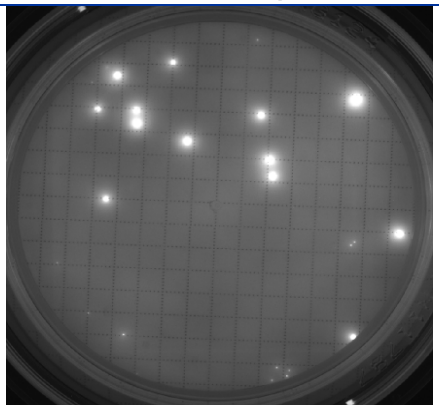
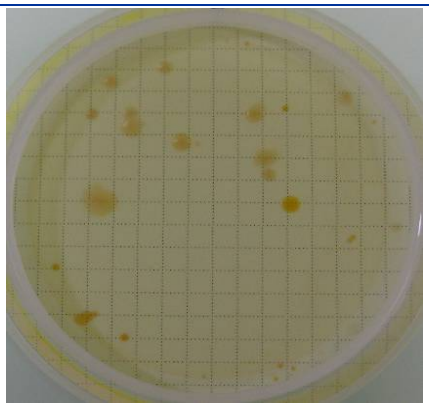
Purified water		Water 1		Water 2	
	Incubation times	**24 hours**	**30 hours**	**24 hours**	**30 hours**
Milliflex® Quantum method	**Milliflex® Quantum camera**				
	Fluorescent count	15	22	31	33
	Fluorescence recovery	50 %	73.3 %	77.5 %	82.5 %
	CFU count after re-incubation	24	23	31	35
	Re-incubation recovery	80 %	76.7 %	77.5 %	87.5 %
Compendial method (7 days)	**CFU count**	30		40	

Table 3: Comparison of fluorescent micro-colonies of a stained membrane with macro-colonies of the same membrane after re-incubation.

Water 1	
Milliflex® Quantum camera picture (membrane stained after 30 hours of incubation)	**Picture of same membrane after re-incubation for 7 days**
	

Results - Time-to-Final Result of Different Water Isolates:

Table 4 contains a list of micro-organisms recovered from different water sources and the respective time-to-final result using the Milliflex® Quantum system as compared to the traditional method. Organisms were recovered anywhere from 24 to 72 hours depending upon the strain.

Table 4: List of customer strains from different water sources and the time-to-final result comparing Quantum and traditional method on R2A media at 32.5 +/- 2.5 °C.

Customer	Water Source	Organism	Quantum Incubation Time (hr)	Traditional Incubation Time (days)
A	Process Water	*Acidovorax delafieldii*	30	5
		Sphingopyxis sp.	30	5
B	Process Water	*Ralstonia pickettii*	24	5
		Deftia acidovorans	48	5
		Methylobacterium fujisawaense	72	5
C	Process Water	*Pelomonas saccharophila*	24 – 30	7
		Variovorax paradoxus	24 – 30	7
		Aquabacterium parvum	24 – 30	7
		Phyllobacterium myrcinacearum	24 – 30	7
D	Purified Water	*Methylobacterium aquaticum*	48	5
		Pseudomonas saccharophila	48	5
		Caulobacter vibrioides	48	5
		Streptococcus sanguinis	48	5

Interpretation:

The results of this study showed that: 1) Using the Milliflex® Quantum System, micro-colonies could be detected after 24 hours, whereas colonies were not yet visible at this time using traditional methods; 2) Water samples from different processes required 24 - 30hr incubation in order to achieve > 70% recovery as compared to the compendial method, and 3) Detected micro-colonies can be recovered by membrane re-incubation after the staining step allowing for further identification using existing ID methodologies.

Depending upon the isolate, the Milliflex® Quantum System reduces the time-to-final result of water-stressed microorganisms by a factor of at least 2 to 3 as compared to the traditional method. Strains that would normally require up to 5 days of incubation are recovered after 24 to 30 hours of incubation.

Dimensions and contrasts of the fluorescent micro-colonies allow an easy visual count through the Milliflex® Quantum Reader, without any background observed on the membranes. The fluorescent count is similar to the colony count of the same membrane after re-incubation.

As the Milliflex® Quantum method is non-destructive, re-incubation of stained membranes enables the identification of water contaminants. As a result, investigations are then simplified, facilitating the implementation of appropriate root cause analysis or corrective/preventive action (CAPA) plans.

The ability to quickly detect and enumerate microbial contamination in water systems allows manufacturers to have better process control, higher product yields, and reduction in time-to-market. The product release can be accelerated and storage time decreased. The result is increased efficiency and cost savings for the manufacturer as well as improved quality assurance of the product.

Further reading and information:
Milliflex® Quantum Rapid Detection System Data Sheet
Milliflex® Quantum Rapid Detection System User

EMD Millipore Corporation, Billerica, MA 01821
Phone: +1 800-MILLIPORE
Fax: +1 800-645-5439
www.emdmillipore.com/biomonitoring

Find contact information for your country at:
www.emdmillipore.com/offices
For Technical Service, please visit:
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