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Application Note

Rapid detection of microorganisms in a gel-based hand wash using a fluorescence-based technology

Earlier bioburden counts of two bacterial test strains using EZ-Fluo™ rapid detection system

The recent pandemic has caused a spike in demand for daily hygiene products, including gel-based hand wash. That is why there is an interest for a rapid bioburden testing method that allows such products to be released to the market earlier.

The fluorescence-based technology of the EZ-Fluo™ system is a convenient and sensitive method for the quantitative rapid detection of contaminants in filterable samples. It can detect microbial contamination up to 3 times faster than traditional plate-based monitoring methods. The rapid microbiological method is based on the universal enzymatic fluorescent staining of viable and culturable microorganisms. The fluorescent staining procedure is non-destructive, allowing microorganism identification following a positive result.

The pharmacopoeias recommend membrane filtration for bioburden testing.[1,2] When using an appropriate test method gel-based hand washing products can be filtered to make them compatible with the fluorescence-based EZ-Fluo™ technology.

The aim of the study was to develop such a filtration method and define the most appropriate incubation time for using the rapid EZ-Fluo™ system with a gel-consistency hand wash. A suitable fluorescence-based rapid bioburden test method was developed using two test microorganisms: *Staphylococcus aureus* and *Burkholderia cepacia*. Both organisms are opportunistic pathogens, with *S. aureus* called out in USP 62 and *B. cepacia* in USP 60.

Material

Hardware, consumables, media, and reagents

Table 1: Hardware used to carry out the study

Item	Cat. No.
EZ-Fit® filtration manifold and pump	**EZFITBASE6 and EZSTREAM1**
EZ-Fluo™ reader system	**EZFKIT001WW**

Table 2: Expendables used to carry out the study

Item	Cat. No.
EZ-Fit® filtration units 100 mL, 0.45 µm mixed cellulose ester	**EFHAW10MS**
Fluid A	**STBMRFA34**
90 mm TSA plates	**1460040120**
EZ-Fluo™ reagent kit	**EZFREAG57**

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Bacterial strains

Burkholderia cepacia ATCC® 25418

Staphylococcus aureus ATCC® 6538

Staphylococcus aureus was chosen as it is naturally found on the skin of half of the human population and also named in the EN 13727-2012 (Quantitative suspension test for the evaluation of bactericidal activity in the medical area - Test method and requirements).[4]

Method and principle of detection

Filtration method

Preliminary trials showed that the untreated hand wash could not be filtered due to its gel-based consistency. Therefore 10 g of hand wash was diluted in 100 mL of Fluid A, a common microbiological diluent.

A filtration unit was aseptically installed on the EZ-Fit® filtration system. 100 mL of sample, prepared as described above, was poured into the filtration unit and filtered. After filtration, the membrane was disconnected from the device and aseptically transferred onto a 90 mm TSA agar plate and the plate incubated at 32.5 °C. The incubation time depended on the method: for rapid detection with the EZ-Fluo™ system the colonies had to be big enough to emit a sufficiently strong fluorescent signal, while after re-incubation or when using the traditional plating method the colonies had to be visible and easily countable with the naked eye.

A microorganism cyrosuspension was thawed at room temperature and serially diluted using NaCl peptone buffer to achieve a concentration of 10 to 100 CFUs in 100 µL.

Staining and reading procedure

The fluorescence detection method is based on an enzymatic reaction. The fluorogenic substrate used is a non-fluorescent viability marker that is cleaved by non-specific ubiquitous intracellular enzymes, resulting in a fluorescent signal. Natural amplification of fluorescence by intracellular accumulation is an indicator of microbial metabolism and thus of living cells. The dye is diluted in a staining buffer to enhance cell membrane permeability. However, the fluorescent stain is diluted due to the take-up of the buffer into the cells.

A liquid media pad is pre-wet with 1.7 mL of staining solution and the membrane transferred from the agar to the liquid media pad inside a small Petri dish. The Petri dish is incubated at 32.5 ºC for 30 minutes. The Petri dish with the filter membrane is then placed into the EZ-Fluo™ reader and the fluorescent spots are counted.

Re-incubation procedure

The optional re-incubation step allows micro-colonies to keep growing for subsequent identification. The membrane is transferred from the Petri dish onto a new agar plate which is then incubated for the remaining standard incubation time.

The incubation time for the rapid method

The appropriate incubation time is defined as the minimum time necessary to achieve a recovery rate higher than 70% of the traditional method count.

The calculation is based on two recovery measures:

- The fluorescence recovery is the fluorescent colony count as a percentage of the traditional method count. It is used to verify that the colonies are detected after staining (reliability of the staining).

$$\text{Fluorescence recovery (\%)} = \frac{\text{(average of fluorescent counts)}}{\text{(average of traditional method counts)}} \times 100$$

- The re-incubation recovery is the count of visible colonies on the stained membranes after re-incubation, as a percentage of the traditional method CFU count.

$$\text{Re-incubation recovery (\%)} = \frac{\text{average of CFU counts after re-incubation}}{\text{average of traditional method counts}} \times 100$$

The optimal incubation time must allow a sufficiently intense fluorescent signal to develop. Both fluorescence and re-incubation recoveries must be at least 70%.

Results

Neutralization of the product's antimicrobial activity

To assess the product's antimicrobial activity, the sample was spiked with a microbial suspension (*Staphylococcus aureus* or *Burkholderia cepacia* between 10 and 100 CFUs) before filtering. No colonies could be observed for either microorganism after conventional incubation, indicating that the hand wash has a strong antimicrobial activity.

To assess the efficiency of antimicrobial activity, neutralization two rinsing procedures were tested: the membrane was rinsed with 100 mL of Fluid A either once or twice.

A single rinsing with 100 mL of Fluid A was sufficient enough to wash away the antimicrobial residues on the membrane and to allow the growth of the microorganisms at the same level as without the product, with a recovery rate of 97.5% determined for *Burkholderia cepacia*. Adding a second rinsing step with 100 mL of Fluid A resulted in the same level of recovery (98.8% for *Burkholderia cepacia*).

The hand wash's compatibility with the EZ-Fluo™ system

Some products may induce a fluorescent background, which can lead to false-positive results and prevent an accurate count of colonies with the EZ-Fluo™ system. Therefore, the hand soap's matrix needed to be tested for any fluorescent background.

A product sample was filtered, and the membrane incubated on TSA agar plates. The membrane was stained and observed with the EZ-Fluo™ reader. TSA agar plates and Fluid A do not generate a fluorescent background, so if there is fluorescent background it must be caused by the product.

However, no fluorescent background was detectable, proving that the hand wash does not generate a fluorescent background.

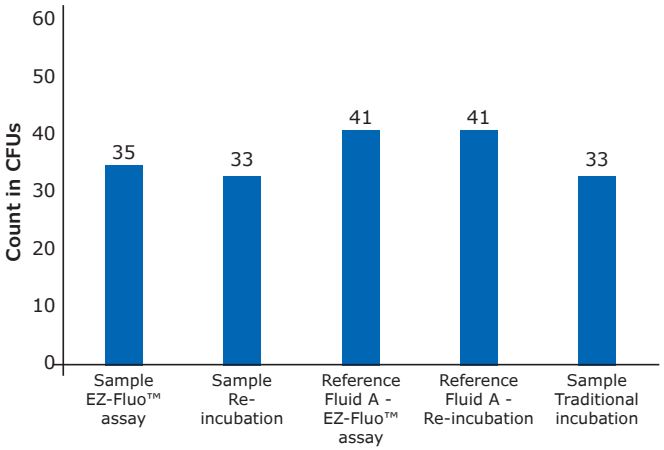
Determination of the incubation time for the rapid method

To determine the incubation time of a plate for the rapid method, the sample was filtered, and the rinse was spiked with a microbial suspension (*Staphylococcus aureus* or *Burkholderia cepacia* between 10 and 100 CFUs). A reference control was also performed using Fluid A spiked with the respective microorganism, but without hand soap.

A rapid incubation time of 16.5 hours for *Staphylococcus aureus* was used based on previous internal studies. (see **Figure 1**).

Note: For each assay, the count in CFUs represents the average of 5 replicates.

Figure 1: Count in CFUs of *Staphylococcus aureus* after 16.5 h incubation and staining for the EZ-Fluo™ assays, and after 21.5 h for the re-incubated plates and the traditional method plates. The hand soap matrix is referred to as Sample.



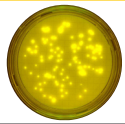
Assays inoculated with *Staphylococcus aureus*

	Sample	Reference Fluid A
Fluorescence recovery (%)	106	**124**
Re-incubation recovery (%)	100	**124**

The sample fluorescence recovery was 106% (sample CFU as a percentage of traditional incubation count) and the sample re-incubation recovery 100%, both far above the acceptance criteria of 70%.

For *Burkholderia cepacia*, the incubation time for the rapid EZ-Fluo™ system was unknown. Since the EZ-Fluo™ system usually reduces the incubation time to between one half and one third of the traditional method's incubation time, and USP 60 stipulates an incubation time of 48 hours of *Burkholderia cepacia* after an enrichment step, durations of 16, 20 and 24 hours were selected for the test. No fluorescent colonies were observed after 16 hours, while after 24 hours, the colonies were too large and therefore difficult to count. After 20 hours, the fluorescent colonies were visible and easily countable. This incubation time was fine-tuned by additionally testing 18 and 22 hours. Some photos of the membrane filters are presented in **Table 3**.

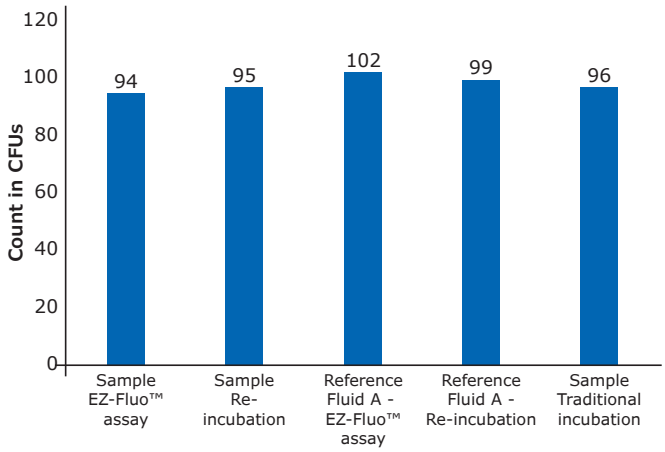
Table 3: Examples of the EZ-Fluo™ system detecting *Burkholderia cepacia* on membrane filters

	Burkholderia cepacia	
	30 °C 18 h	**30 °C 22 h**
Reference Fluid A		
Sample		

After 22 hours, the colonies were already too large but after 18 hours, they had just the right size to be easily counted. Therefore, 18 hours was chosen as the best incubation time for *Burkholderia cepacia* with the EZ-Fluo™ system. The counts are presented in **Figure 2**.

Note: For each assay, the count in CFUs represents the average of 5 replicates.

Figure 2: Count in CFUs of *Burkholderia cepacia* after 18 hours incubation and staining for the EZ-Fluo™ assays, and after 2 days for the re-incubated plates and the traditional method plates. The hand soap matrix is referred to as Sample.



Assays inoculated with *Burkholderia cepacia*

	Sample	Reference Fluid A
Fluorescence recovery (%)	98	**106**
Re-incubation recovery (%)	99	**103**

All assays showed fluorescence and re-incubation recoveries fulfilling the acceptance criteria as stated above for the rapid method. Indeed, the sample recoveries for *Burkholderia cepacia* were 98% for fluorescence-based determination and 99% after re-incubation.

Conclusion

Using the EZ-Fluo™ fluorescence-based technology as a bioburden testing tool reduces the time needed to detect a microbial contamination in a gel-type hand wash product. Under the tested condition *Staphylococcus aureus* can be detected after 16.5 hours and *Burkholderia cepacia* after 18 hours. This represents up to 1/3 of the traditional method's incubation time. Moreover, as the rapid method is non-destructive, each fluorescent microcolony detected can be re-incubated so it continues to grow and yields a visible colony, allowing the identification of the contaminant using any conventional identification method.

The EZ-Fluo™ fluorescence-based technology can therefore accelerate product release and decrease storage times, resulting in financial savings for the manufacturer.

Literature and further readings

1. European Pharmacopoeia 2.6.12 - Total viable aerobic count
2. USP 61 - Microbiological Examination osf Nonsterile of Products: Microbial Enumeration Tests
3. USP 60 - Microbiological Examination of Non-Sterile Products Tests for *Burkholderia cepacia* Complex
4. 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

Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt, Germany

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