BIOMÉRIEUX

Detection of Small Events in Environmental Monitoring Culture Media

The Power of Automated EM Petri dishes Incubation / Reader compared with Visual Inspection

Your Ally in Advancing Quality

PIONEERING DIAGNOSTICS

Laurent Leblanc and Katia Imhoff

R&D, Pharma Quality Control Business Unit, bioMérieux

ABSTRACT

USP chapter <1116>[1] describes the Environmental Monitoring (EM) as a key element to ensure that an aseptic processing area is preserved in an adequate level of control. The quality of the drugs manufactured is directly linked to the capacity to maintain a very low level of microbial contamination. The only technique to understand and follow the contamination evolution of these critical clean rooms is the usage of specific culture media that can recover the environmental flora. Generally, Petri dishes are used to control air and surfaces and cultivate the potential microorganisms found in the classified areas. The common accepted practice for plates examination consists, after an appropriate incubation (temperatures and time), to enumerate discrete colony forming units (CFU). The microorganisms should then grow into distinct macroscopic colonies. The macroscopic scale describes things a person can directly perceive, without the support of magnifying devices. This means that the operators directly observing the Petri dishes should distinguish the presence of the microogranisms with the naked eye. But what is the limit that qualifies a macroscopic object? And at which level of size can we consider the detection accurately? To answer those questions, a standardized methodology to evaluate the performance of manual reading is presented in this study.

INTRODUCTION

Environmental Monitoring (EM) is one of the main microbiological controls that biotechnology and pharmaceutical industries perform. This ensures the safety and efficacy of pharmaceutical products and is a critical control to consider when companies release their finished drug products to market. The historical method is extremely manual, variable and error-prone, but it remains the standard procedure used in the industry for hundreds of millions of samples per year. Indeed, environmental monitoring trend analysis relies on the accuracy of the results recovered on all Petri dishes used in the critical production environments. The analysis is quantitative, the results are expressed in numbers of CFUs. The visual examination of these solid culture media is realized by qualified operators that must comply with the microbiological best laboratory practices described in the USP chapter <1117>[2]. In addition to a solid educational background, an internal training has to be followed to ensure accurate and re-producible results. Theoretically, there is no reason to have discrepancies on results between different trained operators. Standardized results are difficult to achieve due to the natural human variation in sight and evaluation. The huge variety of microorganisms morphology can make their correct detection difficult and providing a correct enumeration is, in some cases, a real challenge. This is illustrated by numerous FDA 483 forms (12) issued between 2011 and 2021 due to bad practices during Petri dishes inspection[3]. Half of them are attributable to counting errors! This questions the accuracy of the results generated during visual inspection of Petri dishes. For this reason, we developed a specific methodology to standardize and measure the quality of detection including challenging events on culture media dedicated to EM.

MATERIALS AND METHODS

- **Calibrated beads**

- Black Polyethylene Microspheres, Cospheric LLC (53-63µm, ref. BKPMS-1.2 53-63um-10g ; 90-106µm, ref. BKPMS-1.2 90-106um-10g ; 212-250µm, ref. BKPMS-1.2 212-250um-10g ; 425-500µm, ref. BKPMS-1.2 425-500um-10g)

- Clear Polyethylene Microspheres, Cospheric LLC (53-63µm, ref. CPMS-1.2 53-63um-10g ; 90-106µm, ref. CPMS-1.2 90-106um-10g ; 212-250µm, ref. CPMS-1.2 212-250um-10g ; 425-500µm, ref. CPMS-1.2 425-500um-10g)

- **Petri dishes and culture media**

A specific medium with no growth performance was poured in a LockSure® Petri dish (bioMérieux proprietary format) dedicated to the environmental control. The formulation was established to prevent the growth of microorganisms on the surface and then allow a multiple reading by several people with the same quality and limiting the occurrence of cross contaminations.

Formulation:

Agar_____ 15g/L

Peptone_____ 14g/L

NaCl_____ 60g/L

Vancomycin_____ 0.1g/L

Amphotericin B_____ 0.1g/L

The Petri dishes were poured with a volume of 30mL. The final aspect of the plates was identical to a regular environmental monitoring culture media.

- **Samples preparation**

The different beads were directly dropped onto the agar plates. The bead size of 425-450µm allowed the handling directly with tweezers.

For the other sizes, beads were, beforehand, introduced in a sterile saline solution and diluted through a cascade of serial dilutions to achieve the expected number of beads in 50µl of solution. The application on the agar was realized with the help of a CMOS image sensor camera (Genie Nano XL® C5100 Color, ref. G3-GC30-C5105 - Teledyne Dasla) positioned on a dedicated backlit bench.

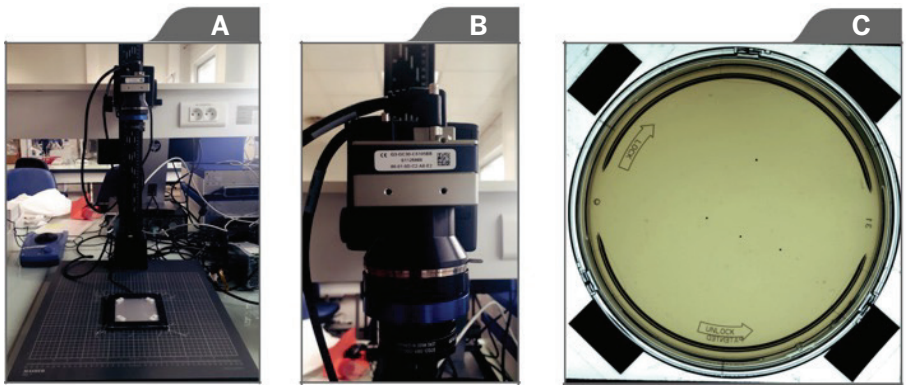


Figure 1: *A. Backlit bench. B. Genie Nano XL® CMOS camera. C. Image of a Petri dish with 4 black beads (425-500µ) in the center of the plate.*

- **Methodology**

A design of experiment was realized to mix the following parameters:

Parameter	Range of variation of the parameter			
Bead Size	53-63µm	90-106µm	212-250µm	425-500µm
Contrast	Strong (black beads)		Weak (white beads)	
Position of the agar	Middle		Edge	
Number of beads	0	1	2-5	6-12

A total of 1,088 plates were prepared. 79% of them were negative and 21% inoculated with the purpose to mimic the higher ratio of negative plates observed in high grade clean room areas.

12 operators from 3 laboratories read independently each plate: 2 labs from bioMérieux (R&D and QC culture media) and 1 Pharma subcontractor lab. All operators were previously trained to enumerate Petri dishes with beads instead of microorganisms. The enumeration was spread over a 5 day period of time to limit a potential impact of tiredness on the quality of the results. The plates examination was realized in the validated routine conditions with the possibility to use black and white backgrounds as well as additional lighting. No magnifying instrument was allowed.

A total of 13,056 results (10,296 on negative plates and 2,760 on positive plates) were generated. For positive results the total amount of data per parameter is explained below:

Number of plates read

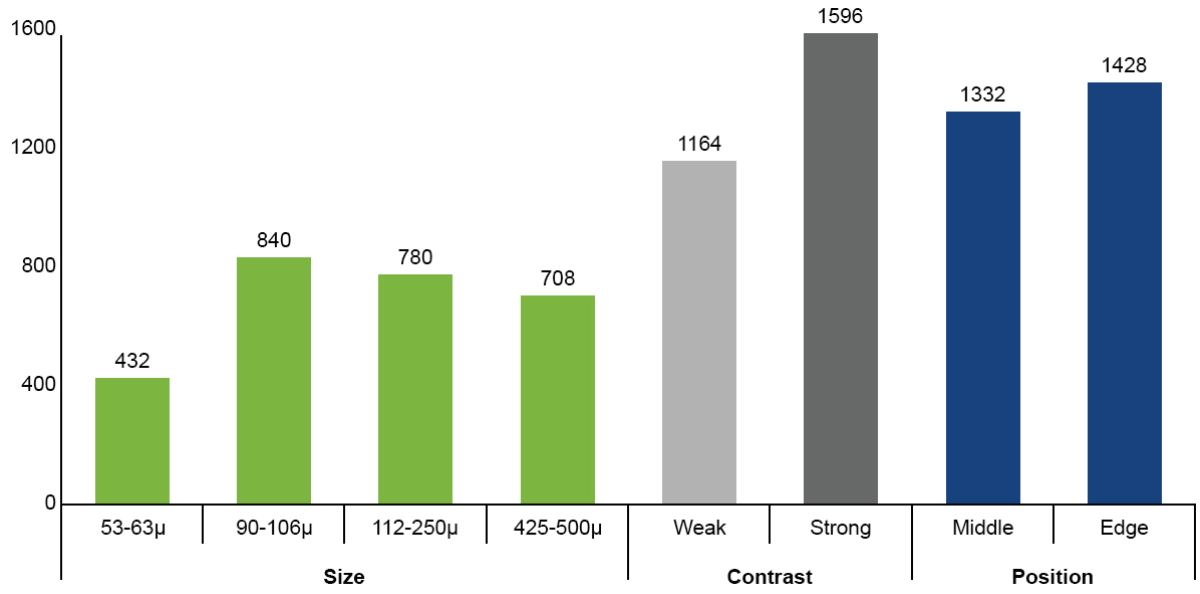


Figure 2: *Repartition of the data generated per type of parameter studied.*

The realization of accurate samples with 53-63µm white beads was impossible even using the high magnifying camera. This specific size and constrast bead was not analyzed in this study.

Analyses were performed using the following softwares: SAS Entreprise Guide v7.1 and Rstudio V1.1.442.

RESULTS AND DISCUSSIONS

- **Analysis at the plate level**

The objective of this analysis is to evaluate the natural perception of the operators during visual inspection and their capabilities to discriminate positive results from negative results.

In this part, only the performance of detection of positive plates (plates with bead(s)) versus negative plates was evaluated, without taking into account the accuracy of the enumeration. The results are expressed as follows:

		Evaluated by the operator	
		Plate +	**Plate -**
Real Result	Plate +	True positive (TP)	False negative (FN)
	Plate -	False positive (FP)	True negative (TN)

The performance of detection per operator is presented in figures 3 and 4.

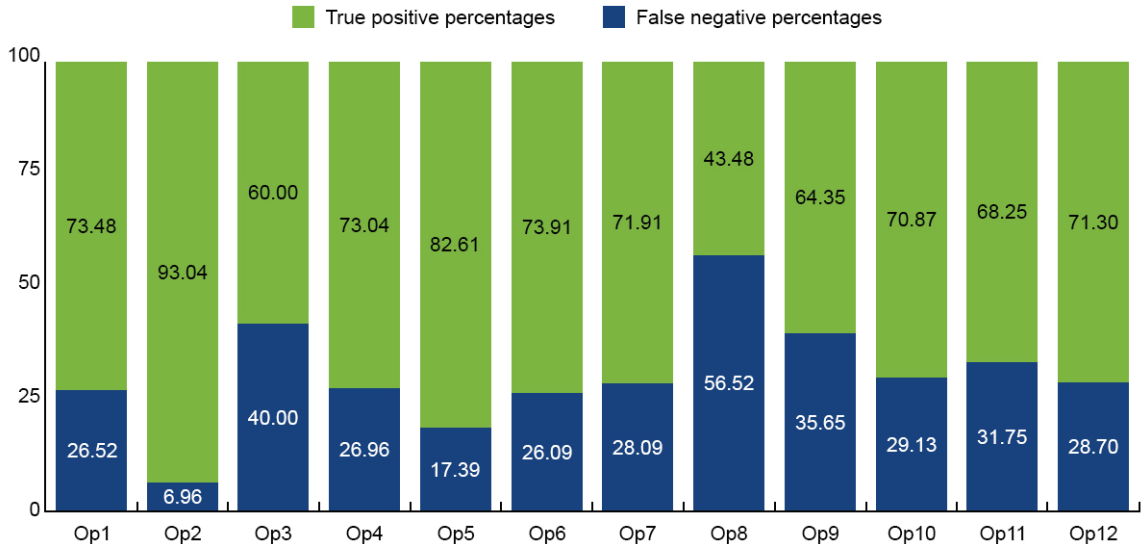


Figure 3: *False negative and true positive percentages measured per operator on positive plates.*

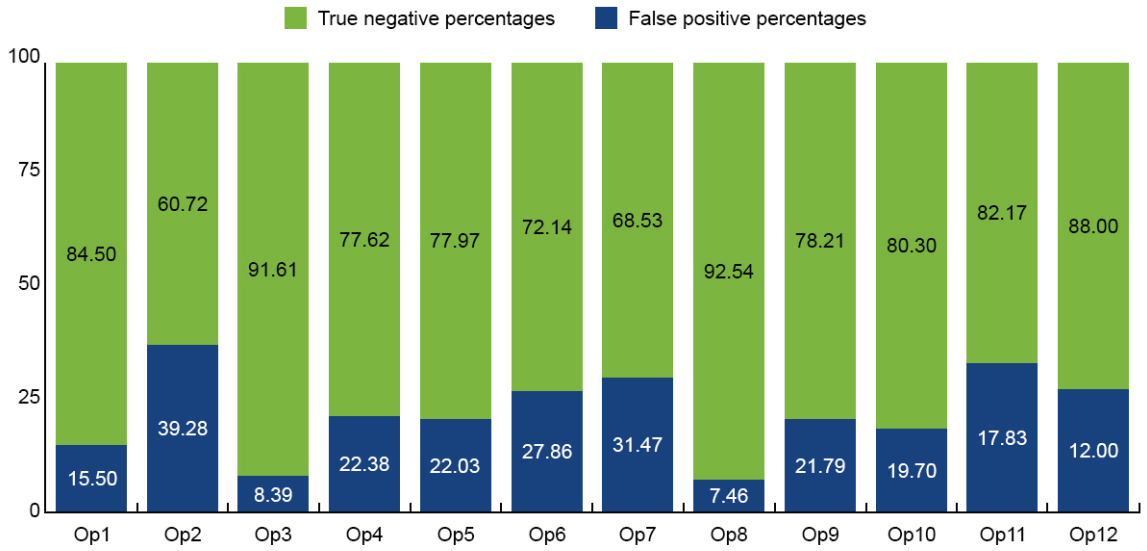


Figure 4: *False negative and true positive percentages measured per operator on negative plates.*

Figures 3 and 4 show a huge variability in the results between the different operators.

This tends to demonstrate that there is a huge level of subjectivity regarding the way a plate is considered positive or negative by an operator. Going deeper in we can classify the operators in 3 types of populations:

1. Operators that generate very few false negative results but at the opposite generate a lot of false positives (Operator 2)

2. Operators that generate a lot of false negatives but very few false positives (Operator 3 or 8)

3. Operators that generate globally the same rate of false negative and false positive (Operator 6 or 7)

A possible explanation of these results could be the difficulty for the human eye to differentiate tiny objects in addition to the subjectivity to assess them as relevant or not. All the plated beads were equal or below a 500µm size that can be considered as very small objects compared to microbiological colonies that can measure several millimeters. In such conditions, the visual inspection on solid media may be impacted by the background noise. Microbubbles, particles, inclusions that are part of the media or that were added during the sampling process may generate confusion between real beads and other particles (like dust).

	Opt 1	Opt 2	Opt 3	Opt 4	Opt 5	Opt 6	Opt 7	Opt 8	Opt 9	Opt 10	Opt 11	Opt 12
Maximum enumeration	8	9	3	12	6	15	11	7	6	10	8	7

Table 1: *Maximum enumeration given by operators on negative plates.*

Table 1 clearly demonstrates that when objects are small, all interfering events can lead to a wrong interpretation. Up to 15 objects have been detected by operator 6 on a negative plate! Even operators from population 2 can detect a high number of false positives.

- **Analysis of the different parameters impact**

This part of the analysis is focusing only on positives plates and evaluates the accuracy of the enumeration provided by the different operators. The results of the 12 operators were pooled together to evaluate the parameters that globally impact the visual inspection.

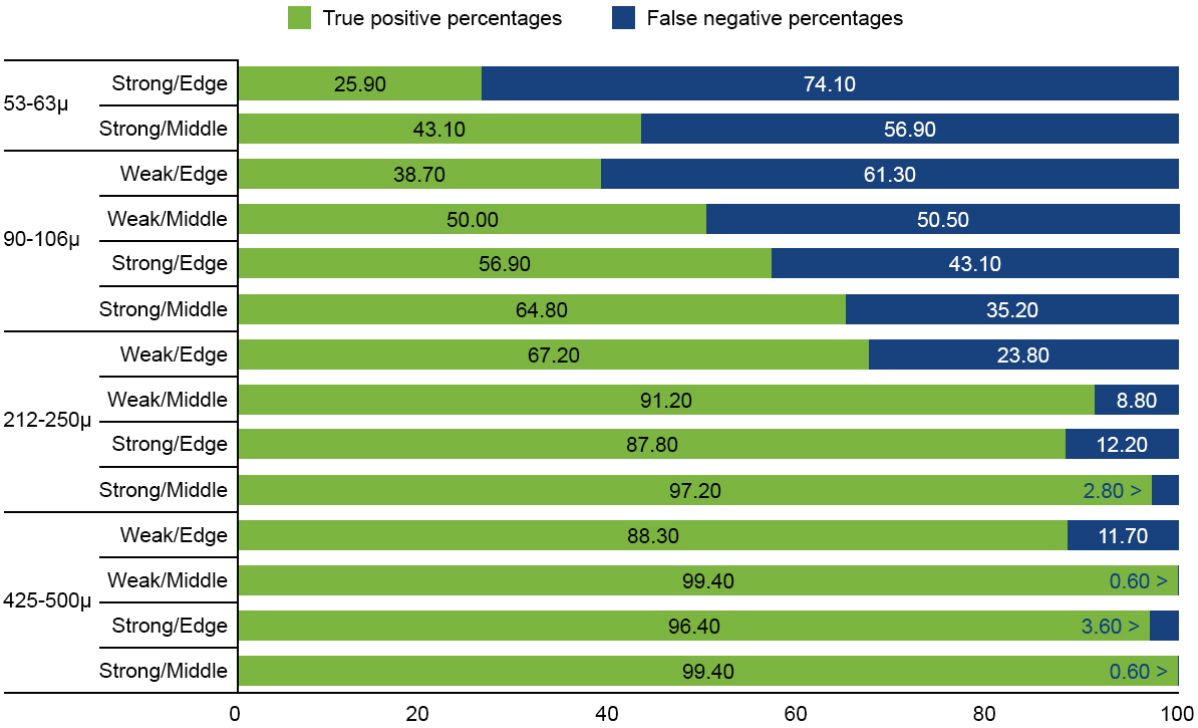


Figure 5: *Cross factors analysis: Size*Contrast*Position.*

Figure 5 shows a clear correlation between the beads size and the performance of detection. The size appears to be the main factor that influences the performance. For the dimension below 212-250µm a huge reduction of the percentage of correct results is measured: from around 90% to below 70%. This negative evolution is increasing with the diminution of the size of the beads.

The second factor that influences the accuracy of the detection is the location of the object on the surface. It is easily visible for the 212-250µm beads size with a weak contrast, depending on the position (in the middle or in the edge) we observe a huge diminution of the percentage of correct results (respectively from 91.20% to 64.80%). This influence of the position also occurs (with a less visible impact) for 425-500µm weak contrast beads with a decrease of the good results percentages from 99.40% for beads in the middle to 88.30% for beads on the edge.

Then the last factor that impacts the detection is the contrast. The contrast influence is less significant than the two previous factors but is present for 425-500µm weak contrast beads. The influence of the contrast on the detection is more noticeable for beads around 212-250µm. It seems logical that the better the contrast, the easier the detection.

CONCLUSION

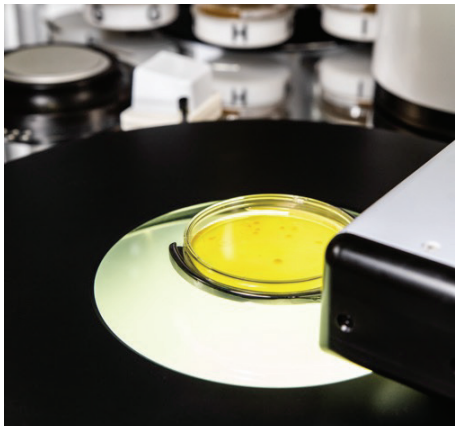
In 2011, Sutton published an article about accuracy of plate counts showing the high variability of the operators and that microbiology itself is a real limit to generate reliable data[4]. In this paper, the correlation between the estimated error rate and the observed low plate count is clearly demonstrated. With the data of our study, we confirm the natural variability of skilled operators that are performing the visual inspection of Petri dishes dedicated to environmental monitoring. In the case of challenging detections, there is a part of subjectivity in the discrimination of small objects. We can then understand the impact of the background noise level on the quality of the detection. Depending on how operators identify objects as relevant or not, the balance of false positive and false negative can be dramatically different. Despite the natural difference of the people performing the EM plates inspection, the study shows common influencing factors. The main one is the size of the beads. Reasonably, we can consider that the accurate limit of detection for the human eye is close to 250µm. Under this threshold, the percentage of missed objects becomes really high (more than 1/3 of false negative results). In addition to the size, the position and the contrast may play a role in the quality of the detection.

Environmental monitoring visual inspection is a manual task, performed by people that have variable performances by nature. Improving the quality of the data from EM results seems difficult knowing this limit. The methodology have to be improved. The Pharmaceutical industry should consider automated solutions, replacing the traditional plate enumeration by automated incubators and plates counters. Furthermore of consistency in the results this technology will improve data integrity and lead to more robust trend analysis.

The 3P® ENTERPRISE solution has been developed to fully address the daily industrial challenges of EM. 3P® ENTERPRISE is a combination of three key products that, when validated and leveraged together, deliver significant value to companies looking to improve their processes.

Thanks to the 3P® STATION, an instrument that automates the incubation and counting of microbiological colonies on EM Petri dishes in real time, pharmaceutical sites are now able to make reliable decisions, maximize efficiency and improve compliance.

A combination of high performance algorithms and high definition images taken every hour mean that there is a standardized reading of plates - no human error - and alerts raised should a sample exceed its specification, allowing rapid corrective actions. The solution is able to deliver significant efficiency gains. Not only are all the manual reading steps, currently undertaken in a laboratory, removed, but as the 3P® STATION can be placed much closer to the manufacturing floor, incubation can start sooner and therefore results obtained more quickly, allowing users to take the right action, faster. The 3P® STATION system has been validated alongside the manual method and provides an end-to-end solution that has been proven to be at least as good as the manual method.



From planning to data reporting, pharmaceutical sites now have the ability to supervise EM control at all stages of the process. 3P® ENTERPRISE is the solution to get faster results, earlier product release as well as faster turnaround of production lines after cleaning validation. As a result: a greater peace of mind thanks to standardized EM control and financial savings.

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bioMérieux S.A. • 69280 Marcy l'Étoile • France • Tel.: + 33 (0)4 78 87 20 00 • Fax: +33 (0)4 78 87 20 90
www.biomerieux.com