EJPPS – European Journal of Parenteral and Pharmaceutical Sciences Volume 27 Issue 2
https://www.ejpps.online/post/automated-and-rapid-microbiology-methods-for-environmental-control-em-and-in-process-control-ipc

Automated and Rapid Microbiology Methods for Environmental Control (EM) and In-Process Control (IPC)

Application of Automated Microbiology for Environmental Monitoring of Clean Rooms

Arnaud Paris - Scientific Affairs Director bioMérieux Industry Healthcare

Corresponding Author: **Arnaud Paris**
Email: arnaud.paris@biomerieux.com

Table of Contents

Executive summary

In order to efficiently control the quality of a pharmaceutical product, it is essential to monitor and analyze the risk of microbiological contamination throughout its production process, both directly during the process as well as when monitoring the production environment. Historically, the qualitative (presence/absence) or quantitative (counting) detection of microorganisms was done thanks to the development of ready-to-use culture media. Even if this type of method remains a reference method and a requirement of regulatory authorities for the release of products, today there are much faster and automated technologies that allow for more efficient and competitive control of production environments and processes. The rise of these rapid and automated microbiology technologies demonstrates the scientific, industrial and economic value of such solutions as a replacement for traditional methods.

Introduction

Heavy investments across the entire bioproduction value chain – from R&D to industrialization and the relocation of production – are behind the growing boom in biotechnology in Europe, to gain competitiveness and catch up with other regions such as the United States.

The UK Life Sciences industry has seen nearly half a billion GBP investment as the Prime Minister works with Biopharmaceutical Industry leaders to drive government, industry, academia and the national health service collaboration in order to achieve a 10 year Life Sciences Vision. Investments include for example £400m from Fujifilm Diosynth Biotechnologies to create the largest biopharmaceutical manufacturing site in the UK; £30m from GSK for research into diseases such as Alzheimer's and to improve the success and speed of R&D of new medicines using advanced technologies, and Bristol Myers Squibb to expand their headquarters for global drug development, among others. The UK's unique ecosystem brings investors access to exceptional talent, reinforced by new training facilities like the National Horizons Centre; health research infrastructure, including translational development sites like Catapult and CPI and a partnership working approach with the NHS. The aim being to cement the UK's status as global science superpower.

In this context, optimizing the control of bioproduction processes and the environmental monitoring of the biopharmaceutical industry is key to supporting the competitiveness of companies and no longer making quality control a bottleneck in the value chain of bioproduction but rather a real added value.

As such, innovative quality control methods have been developed and are implemented more and more routinely and industrially, in particular rapid and automated microbiology methods. Indeed, we observe a movement of the biotechnology industry to move away from so-called "traditional" microbiological controls, mostly based on microbial growth, towards increasingly automated, rapid and integrated technologies. As a result, by freeing itself from traditional microbiology methods, the biotechnology industry can free itself from their high variability and also significantly gain in time to results. Finally, the use of these innovative and digital methods makes it possible to reinforce the traceability and the integrity of the data of the quality control samples.

Intelligent quality control or Smart Quality Monitoring (SQM) (Figure 1) can be applied throughout the production process and environment, whether for bioproduction or the production of Advanced Therapeutic Medicinal Products (ATMP) and Cell and Gene Therapies (CGT).

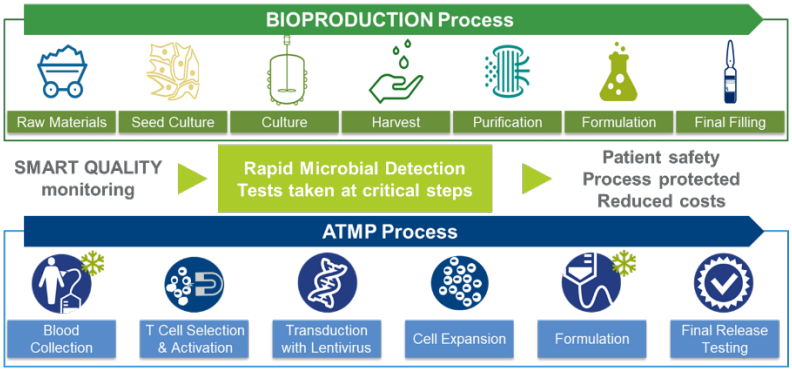


Figure 1. Biotechnology production process diagram (bioproduction or ATMP).

Microbiological controls such as environmental monitoring, bioburden tests, mycoplasma detection, in-process sterility controls, sterility tests on finished products, bacterial endotoxin tests, aseptic process simulations, as well as identifications of contamination etc. are a non-exhaustive part of the Smart Quality Monitoring which can be set up in the pharmaceutical industry.

In routine SQM microbiological controls in bioproduction or ATMP manufacturing, there are 3 major applications for Rapid and/or Automated Microbiology technologies:

- Environmental Monitoring (EM),
- In Process Controls (IPC),
- Finished product controls

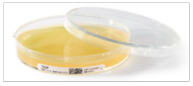
In this article we will focus on demonstrating the value of the automation of EM tests. In a future article, we will then demonstrate the added value of rapid microbiology solutions for IPC.

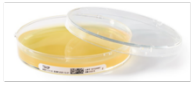
Environmental Monitoring – EM

State of the art

The regulatory requirements for the classification and monitoring of clean rooms are detailed in various reference documents such as GMP Good Manufacturing Practices (EU GMP Annex 1), the FDA guideline "cGMP Guidance for industry", pharmacopoeia chapters such as USP <1116> *Microbiological Control and Monitoring of Aseptic Processing Environments*, ISO 14644 & 14698 standards and Technical Report PDA - TR13 "Fundamentals of an Environmental Monitoring Program". These reference documents describe EM as a key element in ensuring that aseptic production environments are kept free from potential microbiological contamination, while ensuring the complete integrity of sample-related data from collection until contaminants are identified.

For example, one of the key elements of the EM program is to ensure compliance with acceptable levels of microbiological contamination for the classification of cleanrooms. Annex 1 of the GMP (which should be re-published this year) obviously mentions the environmental control of cleanrooms with the classifications of Grade A to D and the accepted limits of contamination (Table 1).



Grade	Air sample cfu/m³	Settle plates (diam. 90 mm) cfu/4 hours [a]	Contact plates (diam. 55mm), cfu/ plate[c]	Glove print 5 fingers on both hands cfu/ glove
A	No growth[b]			
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

Table 1. Classification and acceptable microbiological limits for the different cleanrooms (Annex 1 of the GMP).

The reference method for monitoring this contamination of cleanrooms is to use irradiated culture media plates, making it possible to recover the environmental flora according to the different media formulations. Once the sample has been taken in a clean room and the culture media plate has been transported to the Quality Control laboratory, the inspection of the plate consists, after incubation at appropriate temperatures and durations, in counting the Colony Forming Units (CFU) on the surface of the media by a qualified operator. To be visible and "detectable", microorganisms must grow into distinct macroscopic colonies visible by an operator with the naked eye. EM control and numeration is therefore a visual and completely manual activity, carried out by people with, by nature, variable – although qualified – counting performances.

However, the Annex 1 of the GMPs advocates the use of automation methods and alternative methods for the microbiological control of bioproduction environments.
Thus, it is clearly mentioned that ***"The adoption of suitable rapid or automated monitoring systems should be considered by manufacturers in order to expedite the detection of microbiological contamination issues and to reduce the risk to product. These rapid and automated microbial monitoring methods may be adopted after validation as long as they are demonstrated to be at least equivalent to the established methodology."***

This is why bioMérieux has developed an integrated and automated EM solution, which is perfectly in line with the spirit of Annex 1 of the GMPs.

Current method limitations

The current traditional methods, based on the growth of microorganisms and counting by operators, have very many limiting factors. Both in terms of counting error, safety for the patient, and in terms of data traceability or finally with regard to their industrial optimization, these traditional methods will benefit from being automated.

Numeration errors

In 2011, Scott Sutton published an article on the numeration accuracy of plate counts demonstrating the great variability amongst operators, and demonstrating that microbiology itself was a real limit to generating reliable data (1). In this article, the correlation between the estimated error rate and the low number of positive Petri dishes routinely observed was clearly demonstrated.

In 2018, Laurent Leblanc and his team demonstrated the natural variability of qualified operators who perform visual inspection of EM plates. This study utilized a microbiological model on culture media plates (2).

According to this study, depending on the operators and the detection of the events that they qualify as relevant or not, the balance between False Positives (FP) or False Negatives (FN) can be radically different (Figure 2). In addition to natural differences in people inspecting culture media plates for EM, the study shows several common influencing factors, the main one being the size of the events detected. This study shows that one can reasonably consider the precise limit of detection of the human eye to be 250 µm. In addition to the size, the position on the plate and the level of contrast of the events can play a role in the quality of the detection.

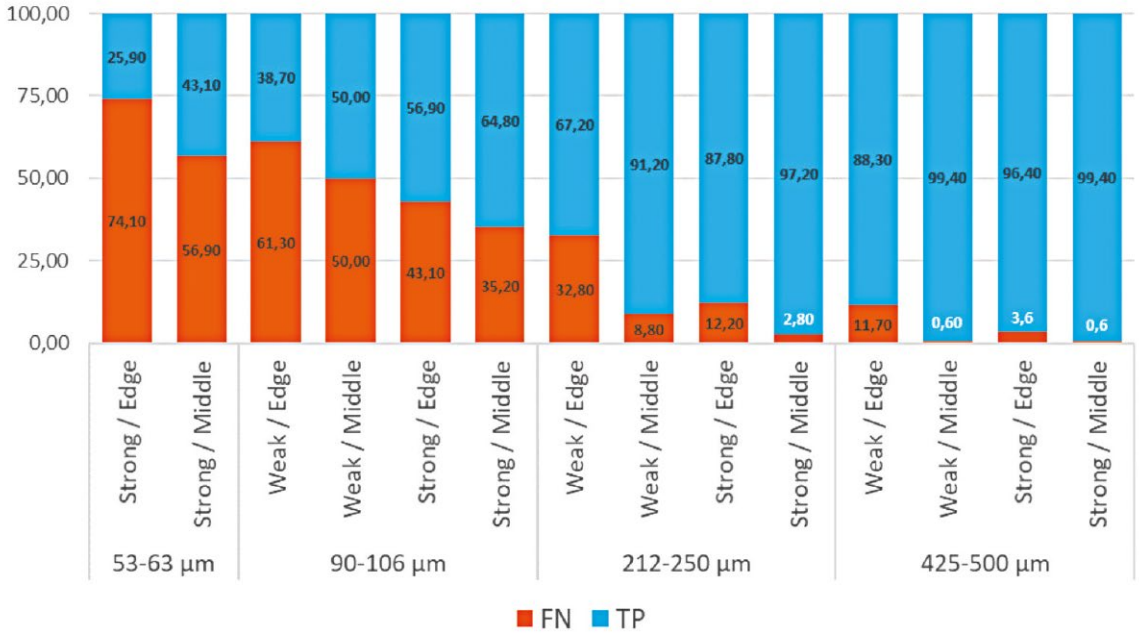


Figure 2. Analysis of the variability of the FN levels from the operators according to the factors of Size / Contrast / Position of the events on culture media plate.

The manual nature and the subjectivity of the human analysis for the detection of small microbiological "events" on culture media plates, can therefore create problems of FP or FN on the counting results of colonies on Petri dishes. The impact can be both industrial and economic in the case of FP, but extremely serious in terms of public health with FN results, if contaminations are not detected in the production environments of injectable products, for example in the controls of Class A environment or in an isolator.

Thus, between 2011 and 2018, among all the FDA Warning Letters about bad practices around the use of culture media for EM, 50% concerned counting errors!

Traceability issues

Moreover, this manual reference method is also subject to many traceability errors, due, amongst other things, to the handwriting of the operators and the manual writing on culture media plates or even on paper. The integrity of the data cannot therefore be completely preserved.

Throughout the whole EM process (Figure 3), many errors can be identified, such as for example:

- Errors in the preparation of labels,
- Operators manual writing error,
- Manual writing on EM plates and / or sampling sheet,
- Use of an unsuitable culture medium,
- Sampling time not respected or not traced,
- Sampling point not respected,

- Use of expired culture medium,
- Culture media plate lost during transport,
- Multiplication of the risks of incorrect information transcription (on the sampling sheets or in any Excel file or LIMS …),
- Incorrect incubation cycle,
- Variable reading performance depending on the operator,
- …

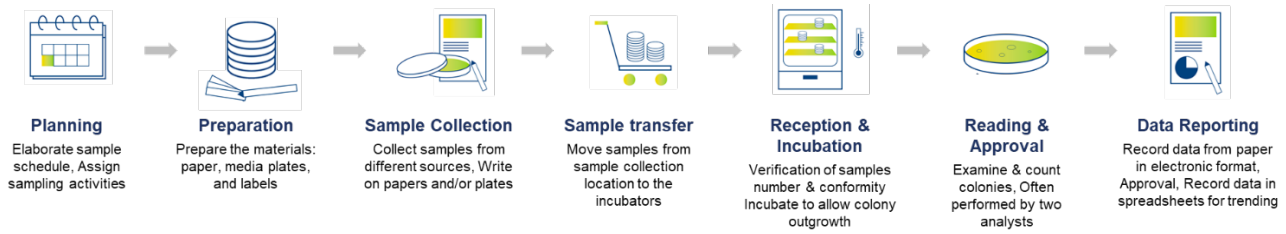


Figure 3. Schematic representation of the complete EM process workflow.

Thus, prior to the publication of the study by Leblanc et al. (2), an analysis of FDA Warning Letters was conducted between 2013 and 2018 (Figure 4): the proportion concerning "Data Integrity" issues increased from nearly 25% to 45%, which over the observed period represented more than a third of the deficiencies noted!

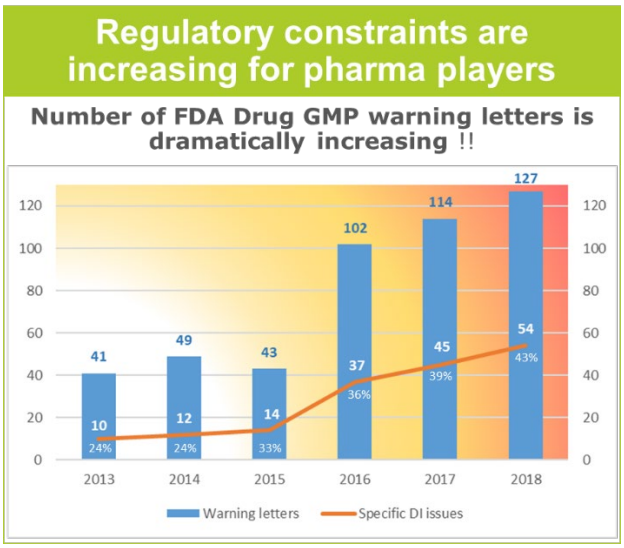


Figure 4. Evolution of FDA "warning letters" and increasing share of "Data Integrity" problems between 2013 and 2018

Industrial inefficiency issues

Beyond possible counting and traceability errors, manual methods also present many operational constraints that make them inefficient from an industrial point of view. Thus, here again throughout the whole EM process (Figure 3), and mirroring the previous risks of error, many sources of operational and industrial optimization can be identified, mainly on the time allocated to low added-value tasks:

- Prepare traceability labels,
- Check the paper "to-do list",
- Manually write on Petri dishes and on paper documents,
- Manually reconcile the quantity of culture media plates,
- Load/unload Petri dishes from incubators,
- Tedious manual counting of colonies,
- Double verification with the "4 eyes" count,
- Writing of manual reports,
- Verification of the information transfer from paper to digital format,
- …

All these limitations throughout the whole EM process can only be overcome with the implementation of digital and automated solutions, such as environmental control software and automated incubation and colony counting systems on EM culture media plates.

Automated microbiological methods for EM

The automation of EM involves mastering the entire value chain of environmental control: from the supply of Petri dishes to the approval of results of colony counting after incubation, the whole data must be traced and controlled to ensure data integrity while limiting the number of human operations. EM automation, to solve those issues, is now possible with completely integrated solutions.

As such, bioMérieux has developed and validated a complete EM management solution: the 3P® CONNECT software is used to manage the planning and traceability of data, and the 3P® STATION system (Figure 5) allows the incubation at different temperatures and automated counting of colonies on Petri dishes.

Figure 5. 3P® STATION automated incubation and colony counting system.

This whole solution was optimized with a panel of pharmaceutical manufacturers (3, 4) – GSK, PFIZER, SANOFI, MSD – who were able to develop it with us towards an operational and industrial design and above all to reach and demonstrate a high level of validated performance equivalent to human analysis.

Counting / numeration errors solving

The high level of performance required for automated counting in order to be equivalent to the human eye requires culture media plates to be read with a kinetic approach during incubation, and not with an endpoint reading, which is a major difference from the classical incubation process and reading step by the operators. This allows, among other things, to reduce the time to result and increase the sensitivity at the same time.

The verification and validation studies were therefore able to show equivalent, if not superior, performances of the automated solution compared to the human eye (5), with full achievement of the parameters set for this technology (Table 2).

Performance Attribute	Population	Metric	Target	Test details
Accuracy & Precision	Artificial inoculations: 10 strains x 2 plate format x 7 inoculation levels x 5 replicates = around 700 plates	Regression line R^2	> 0.95	absolute comparison
		Regression line slope	1	equivalency testing with +/- 0.1 margin
		Regression line bias	0	equivalency testing with +/- 1 margin
Limit of Detection	subset of accuracy dataset	False Negative Rate @colony level	< operator	unilateral z-test @ 95% confidence level
		False Negative Rate @plate level	0	absolute comparison
Specificity	100 strains x 2 plate format x 3 replicates	Percent recovery per strain	> 90%	absolute comparison
	300 plates from aseptic core zones	False Positive Rate @plate level	< 10 %	95% confidence interval

Table 2. Validation criteria for the performance of the 3P® STATION in relation to the numeration on an EM plate by operators.

In order to analyze and validate the performances of the system, the "Reference Count" tool was developed to measure and compare the performance of operators and the 3P® STATION system, and to improve the detection algorithm as much as possible.

"Reference Count" model:
For each culture media plate, counting was performed both by the 3P® STATION system with a kinetic approach (real-time counting during incubation), and with an endpoint reading by operators on the same plates (after complete incubation).

Protocol for defining the "Reference Count":

1) The 3P® STATION gives its definitive count of each culture media plate,
2) Then 3 operators independently count the same plate,
3) The results are reviewed and compared by the 3 operators between the manual CFU counts and the automated CFU counts generated by the 3P® STATION,
4) Based on this comparison and the availability of the colony growth video on the 3P® STATION allowing to "go back in time" for a better visualization of colony growth and mergers, the operators agree on a consensus to define the "Reference Count" for each plate; this allows the counting error to be calculated for the operators and the 3P® STATION at the colony level
5) Then, each plate count result (operator and 3P® STATION) is compared against the "Reference Count", and False Positive (FP) and False Negative (FN) rates are assigned if necessary.

This "Reference Count" then allowed us to evaluate and validate the expected performance levels of the 3P® STATION automated system compared to the operators' counts, in particular on the parameters of **"Accuracy & Precision"**, of the **"Limit of Detection"** (LOD), and **"Specificity"** necessary to validate a quantitative method.

- **<u>Accuracy & Precision</u>**

For the accuracy and precision parameters, all the validation tests carried out on:

- about 10 different strains,
- 7 different concentration levels,
- 2 different Petri dish formats (settle plates and contact plates),
- 5 replicates for each condition,

showed a perfect match between the results obtained (Figure 6) and the expected results (Table 2).

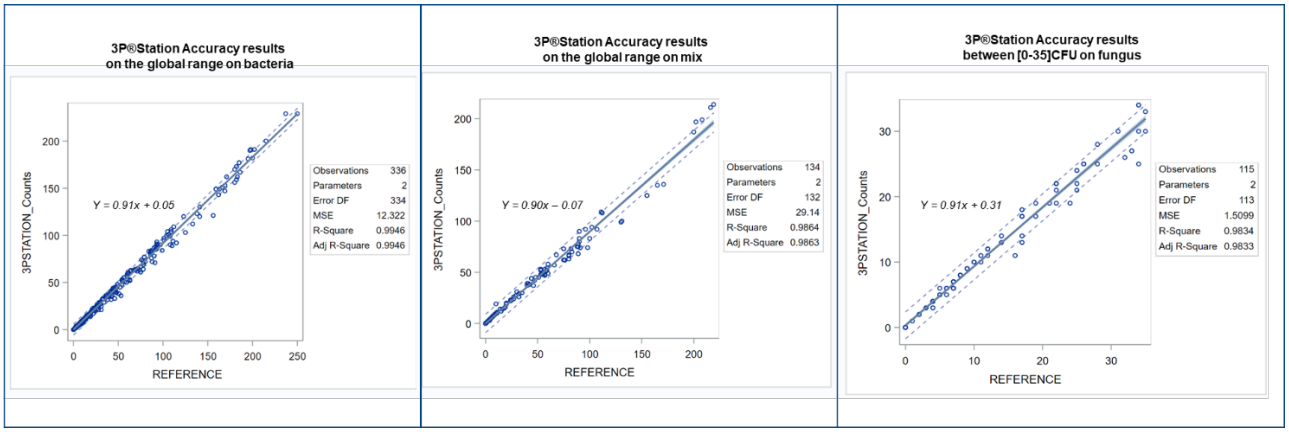


Figure 6. Summary of the validation of the Accuracy and Precision parameters of the automated counting of the 3P® STATION system.

The three parameters of r^2, the slope and the bias have been validated on the different populations of bacteria and moulds as well as on all the microorganisms and on a concentration of 0 to 250 CFU per plate. Validation of the Accuracy and Precision therefore covered a wide window of potential contamination.

- **<u>Limit of Detection / LOD</u>**

For this parameter, two analyses were carried out in parallel on the same data set:

- validation of the FN rate at the plate level,
    - The FN rate per culture media plate represents a possible use of the system as a triage tool to eliminate all negative results – i.e., without microbial growth – by giving a result of presence / absence of colony, without enumeration (for example in the context of the classification and monitoring of Class A clean rooms or according to Annex 1 of the GMP, an absence of growth is expected (NO GROWTH))
- validation of the FN rate at the colony level,
    - The FN rate at the colony level reflects the exact level of counting and reading accuracy against an operator counting each colony on the plate as an individual event.

After a statistical analysis of the data, using the Chi^2 test, the FN rate at the colony level from the 3P® STATION is not significantly different from the FN level of the operators, p-value = 0.78 > 5% (Figure 7), which validates the expected performance criterion on the Limit of Detection.

As for the box false negative rate at the plate level, it is 0%, perfectly in line with the expected level of performance

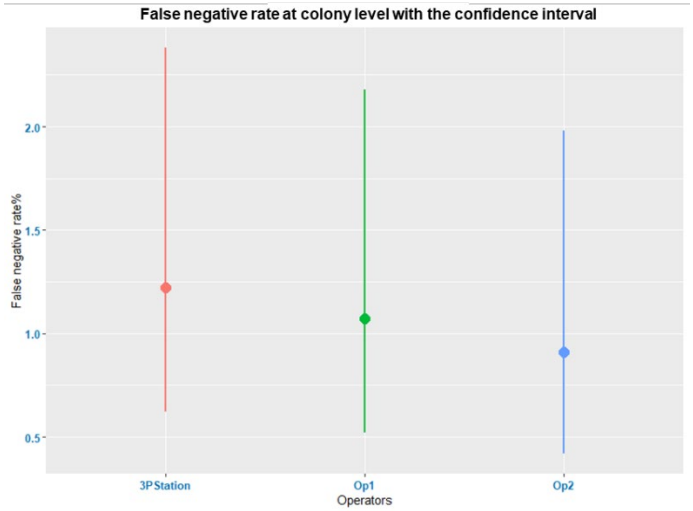


Figure 7. Summary of the validation of the LOD parameter of the 3P® STATION system in comparison with the operators.

- **<u>Specificity</u>**

To validate the Specificity parameter of the 3P® STATION, 300 EM culture media plates were analyzed – 150 settle plates and 150 surface contact plates – the objective being to define the two sub-criteria of FP and Percent Recovery (PR).

The FP rate at the plate level was validated at 0.67% [0.18; 2.41], which is well below the 10% expected for this criterion. This first parameter relating to the specificity is therefore comfortably validated.

With regard to the second parameter on the expected PR by microorganisms (according to the types of microorganisms and the families of microorganisms) greater than 90%, here again, the demonstrated performances comfortably validate this specificity criterion (Table 3).

Microorganims	Recovery% - 90mm	Recovery% - CT
B GRAM -	98	96
B GRAM +	98	93
C GRAM +	98	97
MOLDS	96	91
YEAST	98	95

Table 3. Summary of the validation of the Specificity parameter on the Percent Recovery rate of the 3P® STATION.

The validation of the specificity therefore showed a perfect match between the results obtained and the expected results (Table 2).

Thus, by using the "Reference Count" method, the equivalence of the performance of the 3P® STATION with respect to the numeration of culture media plates by operators could be demonstrated on all the expected performance criteria:

- accuracy & precision,
- limit of detection,
- specificity.

Traceability issue solving

At the different stages of EM control (Figure 3) – Planning, Preparation, Sampling, Transport, Reception & Incubation, Reading & Approval, Data Transfer – previously manual actions can now be automated and digitized through the use of EM management software such as 3P® CONNECT.

Thus, over the entire EM process, almost 40% of the manual and low value-added steps can be eliminated, thereby reducing the risk of information loss and data traceability/integrity issues. For example, editing and sticking labels on EM plates, checking their expiry dates, checking the sampling point, writing and transcribing sampling information, or the many steps of data consolidation and verification are all manual steps with a high risk of error which no longer exist, or which are minimized because they are managed by the 3P® CONNECT software.

The implementation of EM management software therefore allows for superior control of production areas, and improved responsiveness and compliance in the event of an audit. Indeed, the absence of paper documentation, the data integrity, the traceability of all the samples, the possibility of an audit trail (21 CFR Part 11) as well as the tools for trend analysis and issuance of reports are all factors reinforcing the control of EM and its security.

Finally, the possible integration of this solution with an existing and routinely used LIMS makes it even easier to integrate into a global management model for data traceability and supervision of the EM program in real time.

Industrial inefficiency issues resolution

Again, taking into account all the EM steps (Figure 3), one study showed that the time saved could amount to a total of almost 3 min for each culture media plate analyzed (Figure 8). This study was carried out on a manufacturing line and Quality Control laboratory of a production site, and almost 99% of the complete EM workflow was evaluated with the use of the 3P® CONNECT software.

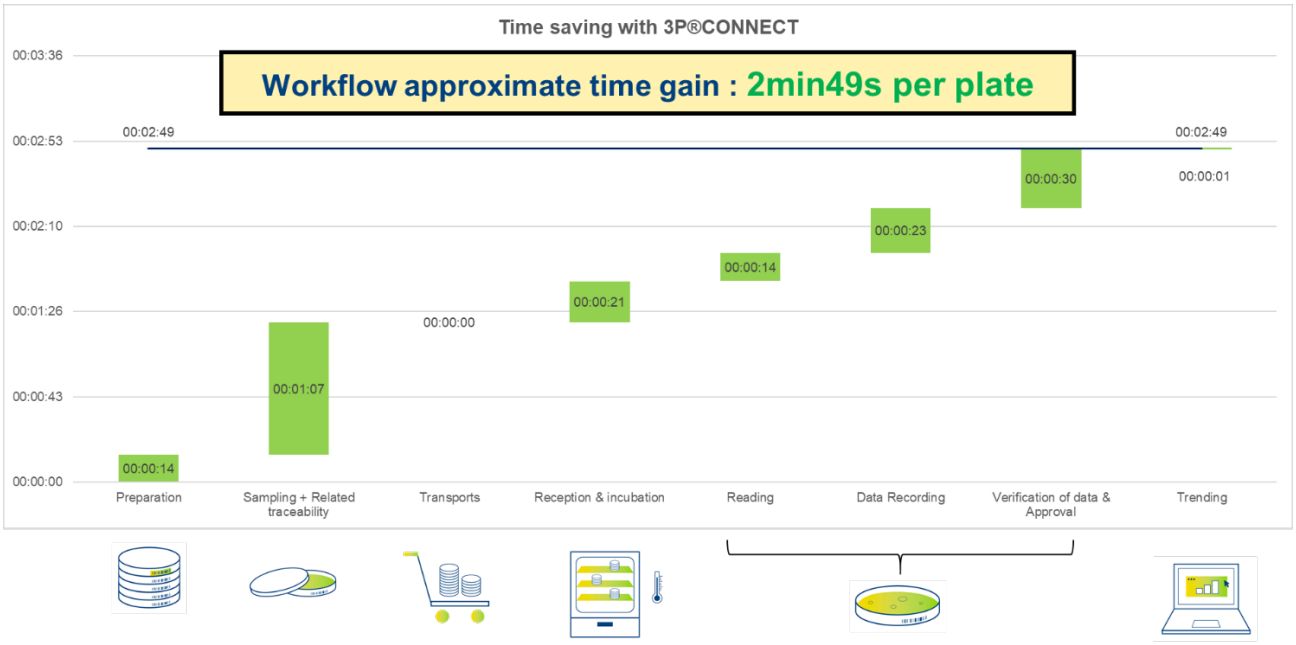


Figure 8. Study of the overall time saving per culture media plate managed and analyzed with the 3P® CONNECT and 3P® STATION solutions.

For EM programs of a few hundred to several tens of thousands of culture media plates depending on the manufacturing site, this time saving can quickly become very significant and further improve the global efficiency of the site.

In addition, automated incubators and colony counters of culture media plates such as the 3P® STATION make it possible to obtain microorganism growth kinetics with automated detection and counting every hour. Thus, instead of waiting several days of incubation to obtain a potential contamination result, early events and growth are detected in real time and therefore more quickly, allowing alarms on detection, instead of waiting the 5 to 7 days of incubation (or even more) of the EM plates undergoing traditional incubation and reading.

Finally, industrial studies are underway on the use of a single incubation temperature and reduced incubation time for EM culture media plates in order to continue optimizing the EM process. The aim is to increase flexibility and efficiency from an industrial point of view (6).

Indeed, a 2017 internal survey of more than 50 biopharmaceutical companies on their practices in terms of incubation of culture media for EM showed that many incubation schemes (number of culture media plates, temperatures of incubation, sequence of incubation, etc.) were used according to the manufacturers. However, it emerges that even if dual temperature incubation is the most common practice (nearly 60%), single temperature incubation is already routinely used in nearly 20% of cases. A similar study by the PDA (7) from 2017 also shows that more than 25% of the biopharmaceutical companies surveyed were already using a single incubation temperature.

So, the automation of incubation at a single temperature and the automated colony counting on culture media plates is an opportunity to potentially reduce the incubation time by 1 to 2 days, on a cycle of 7 days of incubation for example, i.e, nearly 30% reduction in the incubation cycle, which represents a significant gain in terms of industrial efficiency.

Summary on EM

With regard to EM, depending on the production sites, hundreds of thousands of controls can be carried out each year on culture media plates to ensure the contamination control status of all production areas (Classes A, B, C, D). Traditionally, these manual EM controls require waiting for the microorganisms' growth, managing incubations at different temperatures, and counting the CFU on each plate manually. Upstream of these samples and the analysis of the results after incubation, the planning and manual management of the entire EM workflow are also key points which generate constraints of data integrity and issues of traceability of all the data.

This cumbersome process and these methods are subject to many potential errors due to their manual characteristics, such as critical errors of FP or FN during the CFU counting step by Quality Control operators, but also traceability problems and data integrity management issues which are in particular increasingly reported by the FDA.

This is why EM management solutions such as 3P® CONNECT, ranging from sampling planning to automated counting with the 3P® STATION system, are necessary to overcome the problems identified on all the workflow.

These automated and validated microbiology solutions now make it possible to obtain results that are much more standardized and faster than reading by the human eye. The 3P® STATION also makes it possible to manage different incubation temperatures and different incubation cycles to reproduce the same schemes as the current procedures in place. It is therefore not an alternative method but simply the automation of the traditional method in use by various biopharmaceutical manufacturers. It nevertheless allows for faster, more reliable results and better traceability of the information, in particular to launch investigations or preventive / corrective actions if necessary.

Thus, new automated technologies make it possible to overcome variability and drastically reduce the levels of FP and FN results of EM counts, while maintaining the highest level of data integrity and compliance thanks to the digitization of the information.

The biopharmaceutical industry should consider the large-scale implementation of these automated solutions, replacing traditional manual and risky methods.

Acknowledgement:

Warm thanks to all the experts for their key contribution to this article and to the studies mentioned: Laurent Leblanc, Lisa Mallam, Katia Imhof, Clément Dilas, Matthieu Ribon, Laura Bailac, Franck Guilloteau, Laura Arnaldi, Vincent Girousse, Séverine Bascoul, Laura Bailac, Diara White. And particularly thanks to Lucile Plourde Owobi and Thierry Bonnevay.

Bibliography:

1. Scott Sutton, “Accuracy of Plate Counts”, Journal of Validation Technology, Vol. 17 n°3, pp. 42-46, 2011
2. Leblanc et al. “Determination Of The Variability Of Reading Petri Plates Manually: The Influence Of Size, Appearance And Location Of Events On A Petri Plate Are Examined In This Multi-Operator, Multi-Laboratory Study”, PDA Microbiology conference, October 2018
3. Lucile Plourde-Owobi (Sanofi Pasteur) & Arnaud Paris (bioMérieux) “From Variable Operator Numeration To The Standardized 3P® Station Automated Colony Counting On Environmental Monitoring Culture Media Plates”, Juillet 2020, La Vague n°66 - Microbiologie & Eau
4. Lucile Plourde-Owobi (Sanofi Pasteur) & Arnaud Paris (bioMérieux) “Automatisation et digitalisation des contrôles environnementaux : partenariat industriel et synergie R&D pour accélérer l’innovation technologique et renforcer l’intégrité des données”, Les Journées 2020 POLEPHARMA du Biotesting - Enjeux du "Time to Release" des Biothérapies
5. Arnaud Paris “Automation and Digitalization of the Environmental Monitoring Manual Steps”, Pharmalab Alternative and Rapid Microbiological Methods, 24 November 2021
6. Thierry Bonnevay (Sanofi Pasteur) & Laurent Leblanc (bioMérieux), “Suitability of a Single Incubation Temperature for Environmental Monitoring Program”, 2021 PDA Pharmaceutical Microbiology Conference
7. PDA Research: 2017 PDA Aseptic Processing Survey
8. UK Government, News Story 2nd December 2021: UK Life Sciences industry sees nearly half billion investment as PM convenes Biopharmaceutical Industry leaders to strengthen future pandemic response
9. UK&NI, Department for International Trade, Investment Atlas, Healthcare and Life Sciences landing page

. Glossary :

ATMP : Advanced Therapeutic Medicinal Products

CFU : Colony Forming Unit

CGT : Cell and Gene Therapies

DI : Data Integrity

EM : Environmental Monitoring

FN : False Negative

FP : False Positive

IPC : In Process Controls

LOD : Limit Of Detection

SQM : Smart Quality Monitoring