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Case study/Application

Multisite Qualification of an Automated Incubator and Colony Counter for Environmental and Bioburden Applications in Pharmaceutical Microbiology

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Abstract:

Traditional microbiological techniques have been used for well over a century as the basis for contamination testing of pharmaceutical products and processes. With more recent focus on faster product release and concerns around integrity of the test data, new technologies have been implemented to detect and enumerate organisms faster and provide paperless processes to minimize data integrity issues. Manual colony counting technologies, where incubation is performed in a standard incubator and the plate manually transferred to the colony counter for a single read at the end of incubation, have been used for many years to reduce the potential for human error, however, they pose validation challenges due to poor counting accuracy. Colony

counters that automatically perform both the incubation and enumeration functions (multiple enumeration calculations through the incubation phase) have recently been implemented for QC laboratory analytical processes, supporting a cGMP environment. This paper summarizes the findings of eight companies demonstrating the qualification of an automated colony counter technology to perform the majority of microbial tests required for QC, environmental monitoring, bioburden for in process, bulk drug substance and water system testing. Comparable analytical performance and time to result data generated during individual studies at all companies allows the system to be qualified and implemented for cGMP processes while reducing data integrity risks.

Key Words: Automation, Colony Counter, Environmental Monitoring, Bioburden testing, Performance Qualification, Method Validation, contact plate, Rapid Microbial Methods (RMMs)

Introduction

The introduction of automated colony counter technology in pharmaceutical microbiology is becoming more common. Significant improvements regarding data integrity and counting accuracy are the main drivers for this shift from conventional plate reading to automation.
When introducing a new technology, a risk analysis should be performed to ensure that the validation answers the questions posed in the risk analysis. The key technical risks for colony counters are:

1. Will the automated colony counter be viewed as an alternative microbiological test method and subject to full method validation, or merely the automation of the incubation and reading of a traditional microbiological method and hence subject to a reduced verification.
2. Will the colony counter give higher counts due to its ability to detect microcolonies from either small, non-visible colonies or detection of separate colonies that merge and appear to the eye as a single entity during CFU enumeration. Higher counts may require a change of Action/Alert levels.
3. Will automated vision systems generate false positives and cause more action/alert level excursions.
4. If a shorter incubation time (TTR) is selected, will the colony counter miss contamination that would have been seen with the traditional incubation conditions.
5. Will the technology get regulatory acceptance?

These risks are addressed in the paper.

Any viable (and culturable) microorganism that can be captured on the membrane by sample filtration, spread plating, surface contact or impingement during air monitoring, can be detected and enumerated (1-3). Based on these discussions, data will be presented for verification, a position justified by the USP40/NF35 General Informational Chapter <1223> *Validation of New Microbiological Testing Methods* and industry practice as found in the 2013 PDA Technical Report 33 (Revised) *Evaluation, Validation and Implementation of Alternative and Rapid Microbial Methods*. The verification approach has also been used in several successful health authority approvals with both European and USA regulators.

This study will discuss the advanced imaging system in the Growth Direct® System as an example of an automated colony counter, employing data for the three prime applications: environmental monitoring (EM), bioburden for water, in process samples and bulk drug substance (BDS) from multi-company implementations. Data for the time to results determination and method qualification /suitability obtained by those companies for each application will also be shown.

Technical Background of the Advanced Imaging System Used in the Automated Colony Counter

The Growth Direct® System (Rapid Micro Biosystems Inc., Lowell, Massachusetts, USA) is an automated rapid microbial enumeration platform that integrates digital imaging for colony counting, robotic cassette handling, incubation, and software control.

The Growth Direct® System for rapid microbial enumeration comprises two automated incubators handling up to 659 media cassettes. The Growth Cassette products are plastic contact plate style cassettes containing standard compendial growth media.

The Growth Direct® test method requires the presence of a mixed cellulose ester membrane, 0.45-micron pore size, colored black on the surface of the media to improve the signal to noise ratio for the detection system. During the incubation phase, images of each cassette are taken at intervals of 4 hours, allowing organisms and debris that are naturally fluorescent under the excitation blue light (465-495 nm) of the imager to be detected as objects in the green (505-560 nm) spectrum. Analysis of the behavior of objects over the incubation time by the proprietary algorithms of the vision analysis software allows the Growth Direct® System to distinguish and

enumerate the growing colonies from background and debris that do not grow (4). Figure 1 shows the blue excitation light and green emission from colonies being captured on a charge coupled device (CCD). The imaging method does not harm the cells, and as such is a non-destructive method. The micro-colonies can grow into visible colonies for use in subsequent microbial identification.

The Growth Cassette products incorporate standard media varying with the application. For EM two main media options are available, Tryptone Soy Agar with Lecithin Polysorbate (TSA LP80), or added Histidine and Thiosulfate (TSA LP80HT). For product and water bioburden, TSA, Saboraud Dextrose agar (SDA), and R2A media are available, respectively. All media are standard pharmacopeial formulations used in the pharmaceutical industry. The growth promotion is performed as a separate study and is not reported in this paper. Standard site acceptance criteria would be used to verify the media as an approved media.

Regulatory and Compendial Guidance for the Qualification of Automated Methods

USP40/NF35 General Notices 6, *Testing Practices and Procedures,* provides guidance for the use of automated and alternative test methods. 6.20 *Automated Procedures* states, "Automated and manual procedures employing the same basic chemistry are considered equivalent." The statement is equally true for procedures employing the same basic microbiology such as a plate count and the Growth Direct® System. Furthermore, 6.30 *Alternative and Harmonized Methods and Procedures* states that "Alternative methods and/or procedures may be used if they have advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other specialized circumstances. Such alternative

procedures and methods shall be validated as described in the USP40/NF35 general chapter *Validation of Compendial Procedures* <1225> and must be shown to give equivalent or better results." The Pharmacopeia are being updated to relate to changes in microbial methods. These changes and how the new methods are validated are described in USP <1223> *Validation of Alternative Microbiological Methods*. For colony counters, USP <1223> states the following: "There are commercially available enhancements to growth-based methods that allow colonies on solid media to be read more quickly, with substantially less incubation time, than is possible using only the unaided eye. In the implementation of these enhanced methods for the detection of colony growth, only the detection capability of the method requires verification." This statement supports the view that the Growth Direct® System is not an alternative method requiring method validation. The validity of the definition as an automated compendial colony counter is discussed in a technology review article by Jones and Cundell. (5).

Similarly, the PDA Technical Report No. 33 (Revised), *Evaluation, Validation and Implementation of Alternative and Rapid Microbial Methods*, states the following: "Some alternative or rapid technologies may be considered automated traditional or compendial microbiological methods, especially when the results are in colony-forming units (CFU). These technologies may be qualified for their intended use without the need to demonstrate certain method validation requirements as specified in Section 5.0 of the Technical Report. For these technologies, at least accuracy and precision assessments should be performed, in addition to method suitability and equivalence/comparability studies." The view expressed in USP <1223> is fully supported in this industry practice document.

Ph. Eur. 5.1.6, *Alternative Methods for the Control of Microbiological Quality,* does discuss growth-based methods using the presence of endogenous auto-fluorescent molecules and

metabolites such as reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and flavoproteins within microorganisms. The revised chapter continues to view a technology such as the Growth Direct® System within the framework of alternative microbiological methods. Taking the risk-based approach, as recommended in Ph. Eur. 5.1.6, in conjunction with a verification strategy, a simpler approach can be justified.

Validation Approaches

The analytical testing performed by each site was the same but depending on company definitions the name of the test phase varied e.g., Performance Validation/ Qualification/ Verification. The term PQ will be used in this document to encompass all 3 terminologies. The same approach will be used for the Method Validation/Qualification/Verification.

Performance Qualification (PQ)

Performance Qualification has a focus on the system and compares the system colony count to the visual colony count on the same cassette, rather than an assessment of microbial analytical performance.

Because the basis of the PQ test is to verify the vision software's ability to accurately detect and count the range of colony morphologies seen in the QC testing, the media type under the black mixed cellulose membrane is not critical. The same range of colony morphologies exists on all media.

Time to Results (TTR)

The TTR determination can be performed locally or can be performed at a Center of Microbiological Excellence using laboratory spiked samples of stressed and known slow growing microorganisms obtained from other sites from a global organization. The global TTR

can then be set to those organisms and the TTR verified via on site validation during a technical transfer process. It is possible that a local organism may require a longer incubation so that the site can adapt to its local microflora. Water and EM sampling sites with a historical recovery trend should be chosen to give sufficient counts for detection.

The specification used by all sites to determine the TTR is 85% of the slowest growing detected CFU number. This specification arose from the need to define a simple test that would facilitate a faster indication of a contamination issue for trending, as well as the need to recover sufficient organisms to pass the equivalence testing while accounting for the inherent imprecision of the microbiology test. The USP specification in force for recovery at the time was >70% from USP <1227>, which was later harmonized to the current 50-200%. A TTR had to be set to recover >70% of the traditional method accounting for an imprecision in the microbiology test of 15 to 30% CV from USP <1223>. The median value of 85% was therefore selected and has proven to be a robust specification. Depending on company policies, this number can be altered according to the risk assessment performed. Alternatively, an acceptance criterion that requires no significant difference between the Growth Direct® system and visual counts using standard analysis of variance methods can be used.

Method Qualification/Validation/Verification (MQ)

In the MQ phase, tests provide evidence of suitable system and analytical microbial performance. The assessment of precision and accuracy is performed as part of the MQ phase. Studies of the pharmacopeial organisms should include a mixed organism population of 2 or more species and at least 1 environmental organism from the facility, as proposed in TR33.

Precision (Repeatability) shows the variability of the method when analyzed by the same operator from the same sample pool. When tested to a control method a statistical test to compare the two methods variances should be used.

Accuracy compares the CFU obtained by the colony counter against the standard visual plate count for the same sample.

Method Suitability (MS)

Before routine testing can occur, method suitability criteria for individual pharmaceutical ingredients and drug products, must comply with USP <61>, *Microbiological examination of nonsterile products: Microbial enumeration tests*. Method suitability demonstrates the recovery of the challenge microorganisms in the presence of product sample preparation.

Methods

Performance Qualification (PQ) and Performance Verification (PV).

For consistency of data generated most pharmaceutical companies reporting in this paper used the same protocol as defined in the vendors performance qualification (PQ) documents as part of the modular validation. Similar statistical methods were used for the analysis of data but are not described in detail in this paper. However, most sites employed the Two One-Sided t-Test (TOST) for non-inferiority as defined in both the USP Ch <1223> and EP 5.1.6. Examples of the statistical tests using the technology can be found for water (6), bioburden (7) and EM (8).

Performance testing used a challenge microorganism stock culture, freshly grown but no more than five passages removed from ATCC or an equivalent source or reconstituted commercial preparation such as Quanticult™ or BIOBALL®. The test runs were performed with three

replicates of each test organism. The smallest number of organism types that may be used would be three (*Escherichia coli*, fast growing circular morphology, *Aspergillus brasiliensis*, slower growing irregular morphology with hyphae, and *Bacillus subtilis,* fast growing irregular morphology) to cover the main colony morphologies for detection capability by the Growth Direct® algorithm software. A company-specific environmental organism could be added at this phase if the organism demonstrates an unusual morphology. The target inoculum concentration was between 20 and 100 CFU per 100 µL in 20 mL of sterile buffer or water for bioburden testing or in 50 µL directly plated for EM cassettes (lower volume to obtain good distribution without flooding on the membrane surface). For molds such as *A. brasiliensis*, a smaller inoculum < 20 CFU could be used. A summary of the methods used is provided in Table 1. After incubation on the Growth Direct® System at the required temperature 20-25°C, 25-30°C, 28-32°C or 30-35°C for 3-7 days, a visual count was performed for the colonies on each Growth Cassette. The equivalence of the Growth Direct® system compared to the mean of the three analysts' count was performed.

Time to Results (TTR)

The TTR is related to organism type, media type and incubation conditions. Thus, separate studies should be performed for each sampling site and application. EM samples were usually taken from the lower environmental grades C or D. For water samples, larger volumes than routine can be taken to increase cell numbers. Product bioburden samples are often uncontaminated, so organisms likely to be found in the environment or product are used to inoculate the sample. For product bioburden, organisms were included that have been through a stress treatment present in the process e.g., low pH. For EM testing, the samples usually contain

organisms that are already stressed through disinfection, dehydration or starvation. Sample incubation reflects the maximum duration of the visual method, 3, 5 or 7 days. Visual counts of the colonies on the plate were made by 3 analysts on incubation completion. The CFU numbers can vary depending on the day of final visual count, so the final read date needs to be controlled. The TTR was evaluated from system colony counts collected every 4 hours. When plotted against incubation time, CFU counts show a sigmoidal colony detection curve, Figure 2. The TTR can be determined from a defined set of library microorganisms or by testing the sample site and determining the natural growth time for the "stressed" organisms present. The time at which all detected organisms met an 85% threshold of the visual count is the basis of the TTR.

Method Qualification (MQ)

Following guidelines from PDA Technical Report No. 33 (Revised), *Evaluation, Validation and Implementation of Alternative and Rapid Microbial Methods*, at least 6 replicates of each organism were prepared on the Growth Direct® cassette. The same method was used for each of the application types, but featured several variations: organism preparation, membrane filtration for bioburden and water, and the spread plate technique for environmental monitoring cassettes. The Growth Direct® cassettes were incubated and enumerated on the system, then visually counted by the analyst at the end of incubation.

The range of sample points and sample numbers used in the study is given below:

- For water, sample volumes ranged from 0.1 mL to 200 mL depending on water type with 2 to 25 sample points yielding 192 to 600 test replicates.

- For active air, sample volumes ranged from 200 to 1000 liters from 18 to 83 sample points, yielding 54 to 215 samples. For contact plates, 6 to 132 sample points yielded 36 to 216 samples.

For bioburden testing the guidelines suggest the Pharmacopeial organisms, at least one environmental organism and a stressed organism should be tested for each test product type. The stressed cells should represent conditions from the process, e.g., viral inactivation at low pH (3.0-4.0), as performed by some of the companies. Six replicates of each organism were used for in-process or BDS samples. Calculation of the mean and standard deviation of each data set allows a statistical test of the variance, e.g., Chi-square distribution. The same sample data used for precision are used for accuracy determination.

Equivalence is included in the method qualification, which demonstrates results obtained with the test method agree with the compendial method currently in use. Test sample selection is based on relevant species and a sufficient number of organisms for statistical significance. The incubation time for the new method would be the assigned TTR or the compendial duration if the data is to be used to set the TTR. A minimum of 50-100 individual test samples should be taken and analyzed contemporaneously, with the resulting CFU counts compared.

The main function of the media in the bioburden and water applications is to provide nutrients to allow the organisms to grow. In all cases the traditional methods used for comparison studies had been qualified. Other critical functions of the media in the environmental application include:

1. Neutralization of disinfectant residue from surfaces,
2. Ability to capture organisms on a surface and pull them away for growth,

3. Ability to capture organisms in an air flow without adverse dehydration resulting from active air sampling.

Performance of these tests may be comprehensive or based on literature data with a minimal test strategy to verify the published data.

Method Suitability (MS)

Method suitability followed the same method as described for the method qualification, with the exception that the test sample contained the product. The control was a suitable buffer, (Fluid A, PBS etc).

Results

Performance Qualification (PQ).

Results for the PQ phase are shown in Table 2. All sites had acceptable data for the three key organisms. Some sites extended the testing to the remaining USP organisms and historical EM samples with similar acceptable results.

Time to Results Determination (TTR)

Findings of the time to results study are shown in Table 3. From the data shown, the TTR for the EM testing ranged from 36 to 76 hours using a single incubation format and with a wide range of incubation temperatures used, 22.5°C to 32.5°C. Bioburden testing using TSA ranged from 36 to 52 hours depending on company. Water gave the longest TTR using the low nutrient media with results from 44 hours (rinse water sample) but with the majority in the 100 to 116 hours range.

Method Qualification (MQ)

All companies passed the acceptance criteria for accuracy, precision and equivalence for all applications tested, water, product bioburden and EM. For EM equivalence, most companies used both active air and surface contact samples in the method qualification and from a range of room qualities. Where Grade A areas or WFI was used for sample type, there were no false positives found with the testing as all samples gave 0 CFU. For companies that performed specific disinfectant neutralization, active air media dehydration and contact recovery experiments all passed the assigned acceptance criteria.

Method Suitability (MS)

All sites performing MS for in-process and BDS met the required recovery acceptance criteria. This demonstrates the lack of product interference to the fluorescence detection method used.

Discussion

This paper summarizes the results from 8 global companies obtained after qualification and implementation of an automated colony counter system including automated incubation for

routine microbial testing in cGMP manufacturing. The applications cover the main microbiological testing needs of a QC laboratory, environmental monitoring and bioburden testing for water or product and follow the verification approach proposed in USP <1223> for automated colony counters. The rationale for that approach was based on the media types and incubation conditions, as specified in the harmonized pharmacopeia with the only change being the enumeration of an image of the colonies taken by a charge coupled device (CCD) camera and interpreted by software rather than by the human eye. The visual (human eye) approach has been shown in some cases to have wide variability and sensitivity (15). This, combined with the enumeration of colonies at the end of a 3-7 day incubation period, can lead to significant errors in the microbial status of a product or facility, through overgrowth due to false negative counts caused by colonial mergers. The BioPhorum organization have recently published a reference document to cover the 9 steps from evaluation to routine use of any Automated Colony Counter (9).

To verify the performance of the automated colony detection, a simple comparison of the visual colony count versus the colony count obtained by the system can be made on the same test plate. The amount of testing required can be minimized as the possible population of shapes and size that grow are media agnostic e.g. the same colony shapes exist on all media types depending on organism type, and the vision system has been verified at many companies over the last 10 years. Colony colors are not a factor, as the camera only sees a black and white image of the colony fluorescence, so the range of variations is significantly less than with a traditional white light colony counter. As a result, the three basic shapes are specified for PQ testing, fast growing discrete *E. coli*, slower growing irregular shape *B. subtilis* and the irregular growing hyphae of *A.*

brasiliensis. As the software accuracy is independent of media type sold by Rapid Micro Biosystems (colony shapes are not unique to media type), it only needs to be performed once for an application (e.g. bioburden) and can then be transferred to any other application to be implemented with no further PQ work required.

Following confirmation of the accuracy of the technology, the QC laboratory must decide on the incubation conditions for the required test in terms of temperature (single or serial) and time. Most companies have already validated an incubation strategy for their facility that can be transferred directly to Growth Direct® with no further system qualification. However, due to the ability of the technology to detect micro-colonies earlier, many companies opt to determine the TTR for their facility and the microflora found there. As the microflora found in each site may vary (depending on global locality and environmental conditions) and the incubation conditions used may vary as well, it is unlikely that all sites would see the same TTR for each application. The sites contributing to this paper are from East and West USA, as well as Europe, with validations performed at various times of the year where temperature and humidity could affect microbial species present. As can be seen from the data presented here, incubation conditions vary with mean incubation temperatures for EM ranging from 22.5°C to 32.5°C. The different temperatures will each affect growth rates and detection times for the organisms present. Interestingly, all sites showed good recovery using a single incubation temperature rather than the serial incubation strategy (10-13). With bioburden and water testing, the incubation temperature was the same for all sites, 30°C-35°C, but TTR times did differ due to spectrum of microorganisms and local stressors used dependent on process. It is noticeable that the TTR for the water systems is longer than seen for other applications. The use of R2A as a low nutrient

media was standard for all sites but cannot support the growth rates seen by the rich media used in other applications. The original paper by Reasoner et al. (14) suggested that the optimum temperature for water borne bacteria should be 20°C to 25°C, so use of 30°C to 35°C may not be optimal however the higher temperature is specified in the EP. Coupling that information with the adaptation to a very low nutrient environment adds to the slower colony growth rates and TTR, even with the earlier micro-colony detection. The TTR should also be set with the shift pattern in use at the facility. If a one-shift pattern is used, there is no benefit for a sample result to be produced at midnight as no one can react to it. In those cases, a more conservative time can be used so that the result also appears during a following single shift. With a single shift, the incubation windows would be 40 to 48 hours, 64 to 72 hours, 88 to 96 hours and 112 to 120 hours.

A significant benefit for all sites implementing the automated colony counter technology centers on the improvement in data integrity. Recent discussions on the so-called “four eyes rule”, in which every plate count should be verified by a second analyst have not led to general adoption. The PDA TR80 Data integrity management system for pharmaceutical laboratories discussed the secondary review of microbiological test plates, noting that:

> *“Currently a high percentage of the tests conducted in microbiology laboratories are observational, that is, the results (such as a colony count) are viewed and manually recorded on a paper document or in a computer record. Absent an easy, reliable method to verify the recorded data, some laboratories require microbiologists to use second person verification (e.g., supervisor) by physical examination of the test*

plates. Further, the second person verification could be performed as a discrete step prior to approval of the data or combined with the data approval step"

The recent update to USP Ch <1117>, *Microbiological Best Laboratory Practices*, confirmed the benefit for a second read for the critical sterility test but does not recommend its use for other microbial enumeration methods. The use of an automated colony counter and direct transmission of the counts from plate to laboratory information management system (LIMS) database has improved security and negates any need for a second reader. Most sites in this study have a LIMS connectivity to the Growth Direct® System and can cover the most common LIMS platforms available.

Conclusions

The authors believe this review makes a strong case that colony counters such as the Growth Direct® System qualify as an automated system for the incubation and reading of the compendial plate count based on the conditions stated in USP <1223> *Validation of New Microbiological Testing Methods,* and in the industry practice document, PDA Technical Report No. 33 (revised) *Evaluation, Validation and Implementation of Alternative and Rapid Microbial Methods.* As a compendial plate count, the technology does not require full validation as an alternative microbiological method, only verification of the enumeration software. The technology has been qualified for water, in-process bioburden, and BDS testing as well as for environmental monitoring applications in several global pharmaceutical companies, using a shortened Time to Result that is defined by the site-specific natural flora. The technology has been successfully implemented for in-process bioburden testing as part of new drug applications to the FDA and

EMA. Water and environmental testing have been implemented in routine cGMP areas through annual report changes.

Conflict of Interest Declaration

Jason Rose works on behalf of GSK. Any views expressed are his own and he is not speaking on behalf of GSK.

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Note: USP chapters, standard-setting organization methods, PDA technical reports and FDA guidance documents are identified by title, edition and date in the text of the technology review so are not included in the reference section.

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Tables

Table 1. *The Media, Test Microorganisms, and Growth Direct® Incubation Temperature and Time at the Different Study Sites*

Site and Media	**Control microorganism**	**Incubation temperature and time**
Company 1 TSA R2A SDA TSA + LP80	*B. subtilis* *P. aeruginosa* *C. albicans* *A. brasiliensis* *R. pickettii* *S. aureus* *S. epidermidis* (in-house) *Penicillium species* (in-house) *P. glucanolyticus* (in-house) *B. cereus/thuringiensis* (in-house) Mixed culture	30-35°C for 36 hours
Company 2 TSA + LP80	*B. subtilis* *A. brasiliensis* *E. coli*	22.5–27.5°C for 44 hours
Company 3 TSA + LP80	*B. subtilis* *A. brasiliensis* *E. coli* *S. aureus* *P. aeruginosa* *C. albicans* Mixed culture In-house isolates	30-35°C for 44 hours
Company 4 TSA + LP80	*B. subtilis* *A. brasiliensis* *E. coli*	28-32°C for 44 hours
Company 5 TSA + LP80HT R2A	EM: *B. subtilis* *A. brasiliensis* *E. coli* Water: *B. cepacia*	EM: 30-35°C for 44 hours Water: 30-35°C for < 7 days
Company 6 TSA + LP80HT	*S. aureus* *C. albicans* *S. hominis* *M. luteus* *C. tuberculostearicum* *B. subtilis* *A. brasiliensis* *E. coli* *P. aeruginosa*	30-35 °C for 44 hours
Company 7 TSA R2A TSA + LP80HT	*B. subtilis* *A. brasiliensis* *E. coli*	30-35°C for 44 hours
Company 8	*B. subtilis*	30-35°C for 44 hours

TSA + LP80 R2A	*A. brasiliensis* *E. coli*	

Table 2 *Verification of the Equivalency of the Automated Plate Counter and Traditionally Read Plate Counts*

Site	*B. subtilis* ATCC 6633	*E. coli* ATCC 8739	*A. brasiliensis* ATCC 16404	*P. aeruginosa* ATCC 9027	*S. aureus* ATCC 6538	*C. albicans* ATTC 10231
Site 1	Pass	Pass	Pass	Pass	Pass	Pass
Site 2	Pass	Pass	Pass			
Site 3	Pass	Pass	Pass	Pass	Pass	Pass
Site 4	Pass	Pass	Pass			
Site 5	Pass	Pass	Pass			
Site 6	Pass	Pass	Pass	Pass	Pass	Pass
Site 7	Pass	Pass	Pass			
Site 8	Pass	Pass	Pass			

Note Acceptance Specification: Growth Direct® Colony Count ≥85% of the mean colony count by 3 analysts. Organism colonial shape recorded in PQ to verify vision accuracy by site. Greyed cells indicate the organism was not tested during company PQ.

Table 3. Determination of TTR. Acceptance specification, recovery ≥85% of the visual count for the maximal incubation time.

Site and Media	**System Incubation temperature and time**	**Time to Results**	**Comments**
Site 1 TSA R2A SDA TSA + LP80	EM, water and product: 30-35°C for 72 to 120 hours	Product: 36 hours Rinse Water 44 hours EM: 60 hours	Many species recovered >85% much earlier for EM but minimum times will be driven by worst case recovery organism(s)
Site 2 TSA + LP80	EM: 22.5-27.5°C for 120 hours	EM: 36 hours	100% recovery at 72 hours Microorganisms included standard molds and bacteria and in-house molds and bacteria

Site 3 TSA + LP80	EM: 30-35°C for 120 hours	EM 44 hours	TTR is defined by ATCC strains, site-isolates, slow-growers and heat-stressed organisms. Verified with real samples in routine
Site 4 R2A TSA + LP80 TSA	Water: 30-35°C for 144 hours EM: 28-32°C for 120 hours Product: 30-35°C for 120 hours	Water: 116 hours EM: 76 hours Product: 52 hours	Representative purified water samples from site water loop Real air and surface samples from C/D/CNC areas pH stressed ATCC strains on phosphate buffer (without product matrix)
Site 5 R2A TSA + LP80HT	EM: 25-30°C (surface and air monitoring) 30-35°C (personnel monitoring) for 96 hours Water: 30-35°C for 164 hours	EM: 56 hours Water: 116 hours	Microorganisms for EM TTR: environmental samples from surface, air and personnel monitoring Microorganisms for water TTR: *Stenotrophomonas maltophilia* (in-house strain) *Methylobacterium extorquens* (in-house strain) Water samples of different quality
Site 6EM TSA + LP80HT	EM: 25-30°C for 120 hours	EM: 72 hours	Site-isolates from air and surface monitoring
Site 7 TSA + LP80HT R2A TSA	EM 30-35°C for 168 hours Water 30-35°C for 120 hours Product 30-35°C for 120 hours	EM: 52 hours Water: 64 and 108 hours Product: 36 hours	Water TTR varies by facility flora. Bioburden used pH 4.0 stress method
Site 8 TSA + LP80 R2A	EM 30-35°C for 72 hours Water 30-35°C for 100 hours	EM: 68 hours Water: 100 hours	Microorganisms for WFI water TTR were each "stressed" per JP pharmacopeia: *Pseudomonas protegens* (ATCC 17386) *Stenotrophomonas maltophilia* (in-house strain) *Methylobacterium extorquens* (ATCC BAA-2500) Endogenous bacteria were used for purified water (PUW) and pretreatment water systems, using historically highest count sites based on annual trend reports.

			EM used typical 5 USP organisms plus 3 in-house organisms.

Figures

Figure 1. Schematic of the detection of the autofluorescence of micro-colonies. Top - Microorganisms fluoresce under blue light and their location on the membrane captured by the CCD detector. Middle - Images taken at 4-hour intervals and an increase in a fluorescent object size or brightness confirms a growing colony. Bottom - Accuracy of the system can be shown by comparison to human counts at the end of incubation.

Figure 2. TTR curves for 5 pharmacopeial and 7 environmental organisms, namely Staphylococcus epidermidis, Micrococcus luteus, Kocuria rhizophila, Bacillus pumilus, Brevundimonas diminuta, Candida tropicalis, Aspergillus fumigatus are incubated and imaged every 4 hours for 72 hours at 30-35°C. The cumulative percentage of emerging colonies is shown on the y-axis.

Patented technology uses a blue light causing the micro-colonies to autofluoresce: this is captured on a CCD chip

Powerful software starts to detect colonies within hours, enabling real-time enumeration of organisms

An A. brasiliensis Microcolony in CHO cells

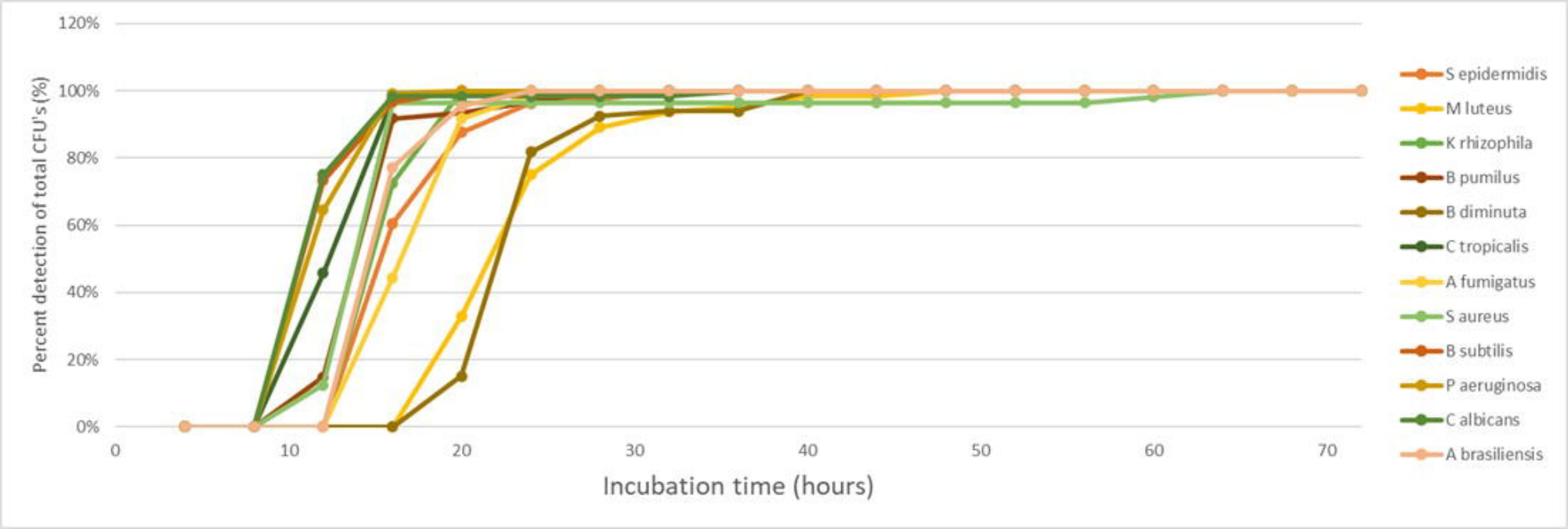
12 hrs | 16 hrs | 20 hrs | 24 hrs | 28 hrs | 32 hrs

The Growth Direct™ counts the same colonies in half the time of the traditional method.

Growth Direct™ Imaging

Visual Plate Counting

Day 1 | Day 2 | Day 3 | Day 4 | Day 5


120%
100%
80%
60%
40%
20%
0%
Percent detection of total CFU's (%)
0
10
20
30
40
50
60
70
Incubation time (hours)
S epidermidis
M luteus
K rhizophila
B pumilus
B diminuta
C tropicalis
A fumigatus
S aureus
B subtilis
P aeruginosa
C albicans
A brasiliensis

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