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A Rapid and Nondestructive Method for Microbiological Testing in Pharmaceutical Manufacturing

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Improving the performance of pharmaceutical microbiological quality control (QC) testing is often included in the goals of the FDA's Process Analytical Technology initiative. That a nineteenth century microbiologist would recognize the methods used by twenty-first century pharmaceutical manufacturers to detect and enumerate microbial contaminants, however, testifies to the glacial pace of technical change in microbiological QC. The persistence of the dominant detection technology, microbial culture, stems in large part from its numerous advantages. Culture tests use low-cost materials, minimize regulatory risk (they are the regulatory "gold standard"), demand low-level skills, deliver high sensitivity for culturable cells, yield pure cultures that can be used to identify contaminating microbes, and are useful for all of the key microbiological QC tests. But culture methods also bring some expensive drawbacks. Slow testing turnaround times (due to extensive cellular replication required for visual detection) are particularly costly to the industry. Lengthy testing cycles increase the costs of held inventory, the amount of discarded product, and the time required to get manufacturing plants up and running after a contamination event. Traditional culture methods can be subjective and require manual processing, laborious documentation, and cumbersome procedures to ensure regulatory compliance.

Over the last decade, several new microbial detection technologies designed to save costs by improving test turnaround have sought to gain a foothold in the pharmaceutical QC market.[1] However, these technologies—which are based on adenosine 5′-triphosphate (ATP) bioluminescence, solid-phase laser cytometry, and flow cytometry—have achieved only spotty success. The hesitant uptake of these technologies derives in part from the complexities encountered when validating methods that deliver numerical results that are very different from and hard to reconcile with the microbial culture test gold standard. Furthermore, some of the new methods fail to address the full range of testing applications because they are nonquantitative, lack sensitivity, or are constrained to small sample volumes. Finally, because these new technologies are destructive (i.e., they generally kill the detected microbes), they cannot generate the pure cultures required as input to microbial identification systems.

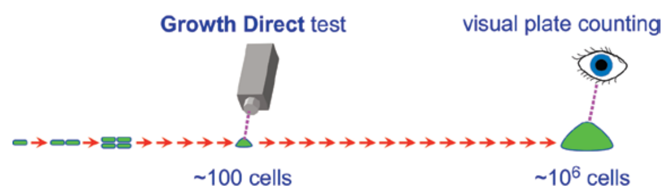

Figure 1 Like conventional plate counting methods, the Growth Direct system detects colonies of replicating cells. By using digital imaging of microcolony autofluorescence, however, the test detects microcolonies at an earlier time than visual plate counting.

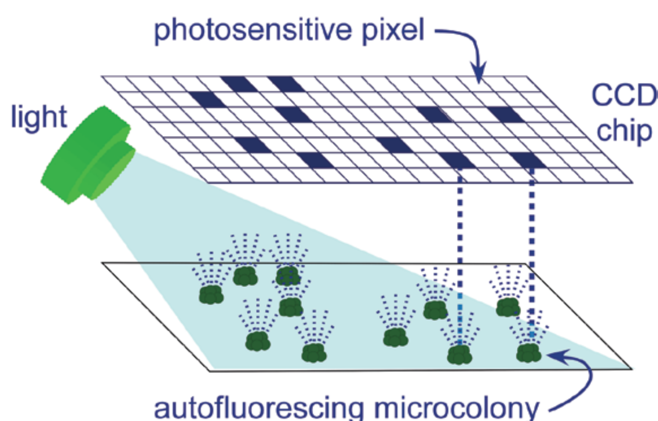

Figure 2 Detection of the native fluorescence (autofluorescence) of the cells in microcolonies. A short pulse of blue light causes each microcolony to emit yellow-green fluorescence, which results in a countable spot on a photodetector array.

The goal in designing the Growth Direct™ system (**Rapid Micro Biosystems**, Bedford, MA) was to address the chief liabilities of the culture test while retaining its advantages. Thus, the system substantially improves test turnaround and automates analysis and documentation while maintaining ultrasensitive detection of replicating microbes, nondestructive detection, applicability to a broad range of tests, and compatibility with microbial identification. The system is congruent with

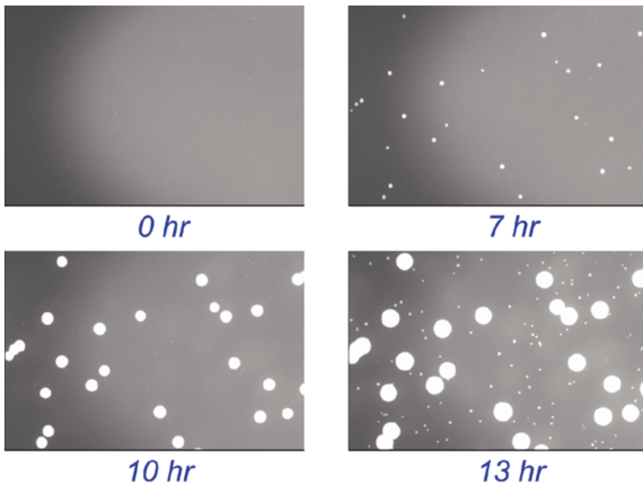

Figure 3 By imaging over time, the Growth Direct system discriminates between growing microcolonies and inanimate fluorescent debris. The figure shows an image time series of a mixed culture of E. coli (faster growing) and B. cepacia (slower growing) microcolonies.

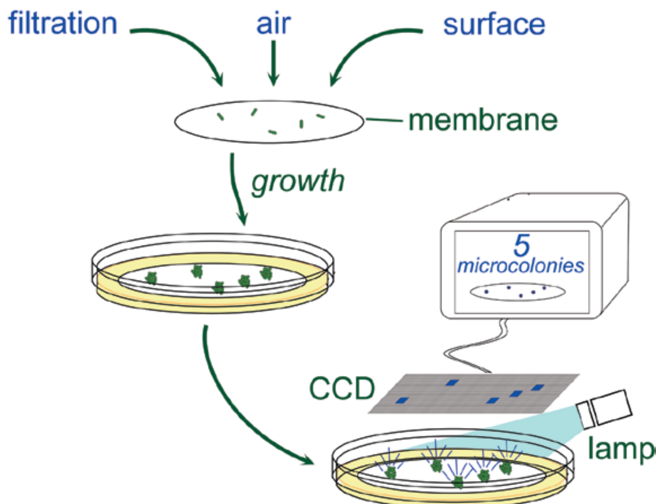

Figure 4 The workflow of the Growth Direct system is similar to that of visual plate counting. For clarity, the diagram does not show the system's optical or mechanical components.

the approved regulatory methods, being based on the same procedures and principles as visual plate counting. System validation is simplified because the test enumerates the same target objects as the current culture test (colonies of replicating cells), and therefore generates results that are numerically equivalent to the regulatory gold standard. The system is currently entering the final stages of product development.

System operation

Detecting autofluorescent microcolonies

Like the current visual plate counting method, the Growth Direct system enumerates colonies derived

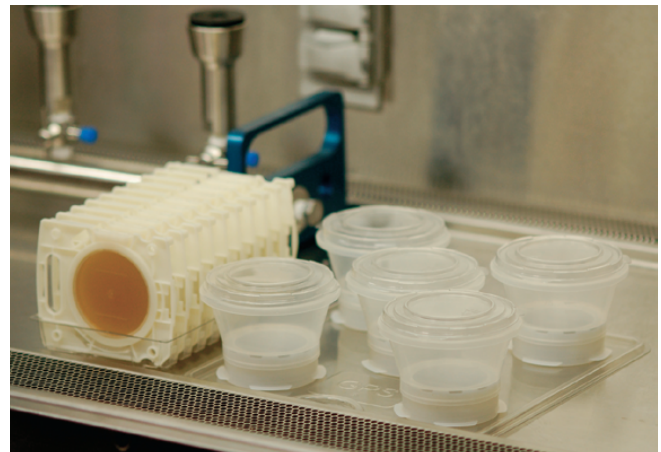

Figure 5 The consumables provided for testing liquid products and raw materials using the Growth Direct system are similar to those used in today's tests. Left: sample cup with membrane filter. Right: growth cassette containing nutrient agar.

from the microbial cells that can replicate on nutrient agar (*Figure 1*). The system, however, uses digital imaging to "see" the colonies much earlier. The technology detects the yellow-green fluorescent signal emitted by the microcolonies when illuminated with blue light (*Figure 2*). Cellular autofluorescence in this spectral region is a property of all microbial cells due to the presence of ubiquitous fluorescent biomolecules including flavins, riboflavins, and flavoproteins. The system's autofluorescent detection technology enables enumeration of all of the types of microbes detected by the current plate counting method. Furthermore, it does not harm the growing cells and thus allows microbiologists to isolate and identify microbes.

Monitoring growing microcolonies

Nondestructive detection enables the Growth Direct system to accurately distinguish growing autofluorescent microcolonies from inanimate fluorescent debris. By imaging at various times during incubation, the system constructs an image time series like that shown in *Figure 3*. The system's image analysis software then builds a history of each fluorescent object over time. The software reports the number of growing microcolonies and ignores nongrowing fluorescent debris.

Workflow and automation

Sample preparation and the upfront workflow for the system follow current practices and thus will be familiar to microbiologists (*Figure 4*). Liquid samples, for example, are prepared as they are today, by pouring the sample into a funnel, filtering through a membrane, and then placing the membrane on nutrient agar in a growth cassette (*Figure 5*). Next, the user introduces a set of growth

Figure 6 *Stacks of growth cassettes are transported in carriers.*

Figure 7 *Growth Direct system.*

cassettes, stacked in a convenient carrier (*Figure 6*), into the Growth Direct system (*Figure 7*). At this point the system takes over, completely automating the process, including performing iterative cycles of imaging and incubation to build the image time series, analyzing the images, and posting the results in reports. Internal system modules enable the concurrent automated analysis of hundreds of samples. Two incubators, which can maintain different temperatures, have a combined capacity for 378 cassettes. A robotic arm transfers the cassettes between the loading racks, bar-code reader, incubator, and imager.

Speed, sensitivity, and accuracy

Time savings

The system detects colonies after only a few cellular generations, long before they become visible by eye. For example, while visually detectable bacterial and yeast colonies have about 5×10^6 and 2×10^5 cells, respectively, the Growth Direct system detects the same colonies when they contain only about 130 and 10 cells. The time-savings for slow-growing microbes can be measured in days or, in certain cases, weeks (*Table 1*).

Accuracy at low levels of contamination

Like visual plate counting, the Growth Direct system features ultrasensitive detection of cells capable of

Table 1 Time-savings for slow-growing microbes using the Growth Direct system

	Mean time to detection (days)		
Species	Growth Direct	Visual[b]	Days saved
Methylobacterium extorquens[a]	2.6	17	14.4
Aspergillus versicolor[a]	1.5	3.6	2.1
Deinococcus proteolyticus[a]	1.6	4.0	2.4
Ralstonia picketii[a]	1.1	3.0	1.9
Clostridium sporogenes	0.6	1.8	1.2
Aspergillus niger	0.8	2.4	1.6

[a]Microbial isolate recovered from a failed sterility test of a pharmaceutical product.
[b]Mean time to detection in broth culture of samples inoculated as described in Figure 12.

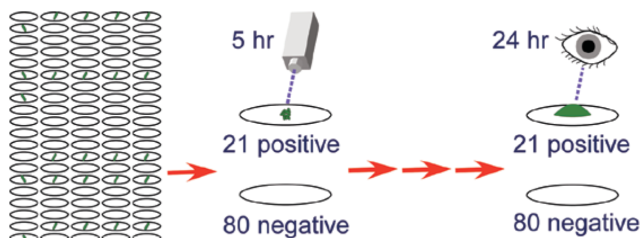

Figure 8 An experiment showing that at the lowest levels of contamination, there is 100% agreement between the number of E. coli colonies enumerated by the Growth Direct system and the visual plate count.

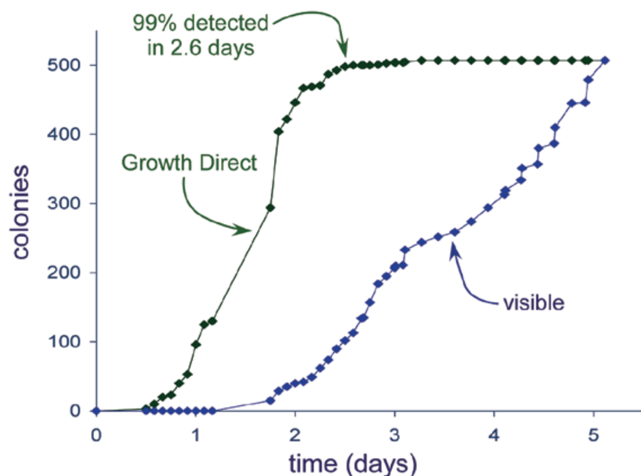

Figure 9 Microbial enumeration of a water sample from a pharmaceutical facility using the Growth Direct system. The rapid method saved about 2.5 days when detecting the same colonies that were detected in 5 days by the traditional visual plate counting method.

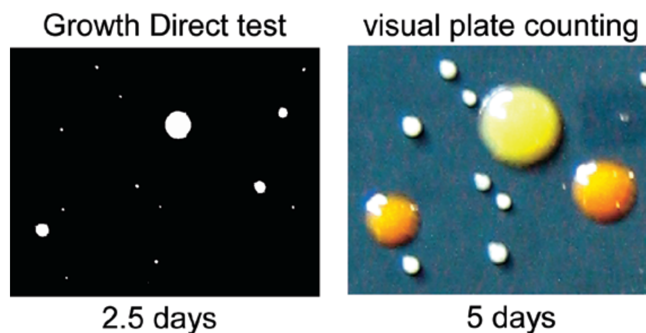

Figure 10 Images of a section of a growth cassette containing a water sample from the experiment plotted in Figure 8. The number and pattern of microcolonies detected by the system at 2.5 days match the number and pattern of the visible colonies seen at 5 days, showing the comparability of the two detection methods.

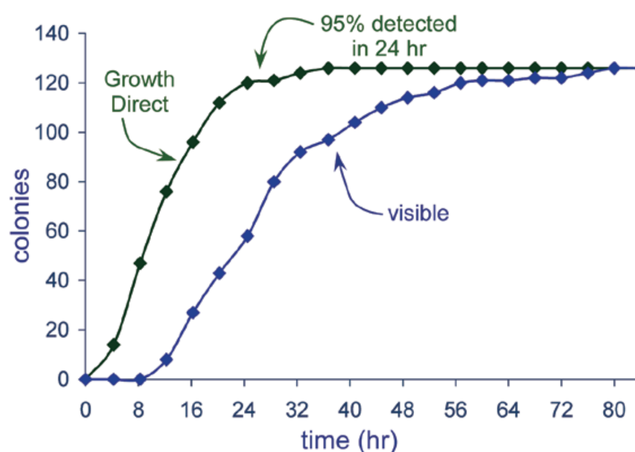

Figure 11 Detecting bacteria in air samples using the Growth Direct system. The system detected in 24 hr the same colonies that required 48 hr to detect using the traditional visual plate counting method.

replicating in culture. Unlike other rapid methods, however, the system facilitates the validation of accurate performance at the lowest possible levels of contamination. For example, a small number of cells can be deposited on a large number of membranes so that most membranes have zero cells and a minority of the membranes have only a single cell (*Figure 8*). One analyst uses the system to score the membranes for those that have microcolonies and those that do not. The membranes are then reincubated until the colonies become visible. A different analyst, in a blinded experiment, scores the same membranes for visible colonies. In experiments using various types of microbes, the Growth Direct system results agree completely with the traditional method, demonstrating accuracy at the lowest levels of contamination.

Rapid testing for key pharmaceutical QC applications

The Growth Direct system addresses all of the key QC microbiology tests. It is no surprise that the method's versatility matches that of the reigning microbial culture technique, given that they are based on the same method principles. A few representative examples, described below, illustrate the scope of the Growth Direct system.

Rapid water testing

Enumerating microbes in the various grades of water constitutes an important element of any QC program in pharmaceutical manufacturing. *Figures 9* and *10* show that the Growth Direct system detects in about 2.5 days the same microbes that require 5 days to detect using conventional visual plate counting.

Rapid environmental monitoring

Careful monitoring of microbes in the air and on surfaces is essential for ensuring that manufacturing processes are under control. *Figure 11* shows that the system detects, in about half the time, the microbes that become visible at the end of the current tests.

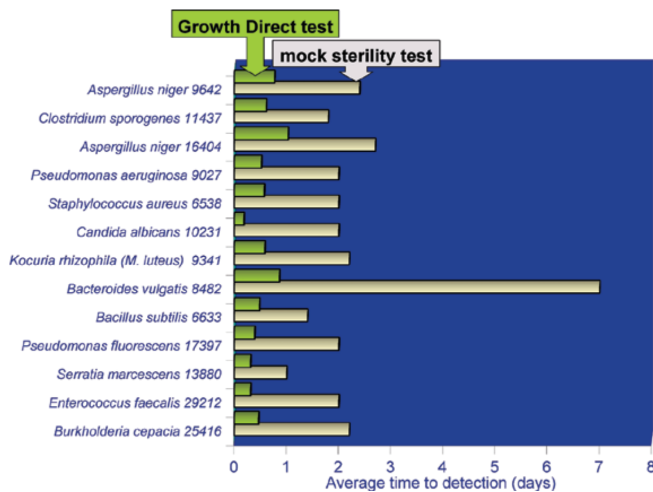

Figure 12 *Comparing Growth Direct time to detection for standard USP test microbes to that of the broth-based sterility test (USP chap. 71). Mock sterility tests (20 bottles for each strain) were inoculated with approx. 0.5 CFU per bottle, incubated for 14 days, and scored daily for turbidity.*

Rapid sterility testing

Sterile products, such as injectable drugs, must generally pass a lengthy 14-day test before they can be shipped. Reducing the time to result for sterility tests could potentially save a manufacturing facility millions of dollars per year. Case studies using the Growth Direct system (see *Figure 12*) show the feasibility of the rapid method for sterility testing.

Summary

The Growth Direct system addresses the major limitations of the current culture-based testing paradigm while preserving the strengths of the traditional methods. In contrast to today's culture tests, the method decreases the time to result and automates much of the process. Like the current method, however, the Growth Direct system minimizes some of the hurdles of regulatory validation because it is so similar to the regulatory gold standard. Because it is nondestructive—in contrast to many other rapid methods—the test is compatible with standard microbial identification systems. Finally, the system addresses the need for rapid feedback on quality throughout the manufacturing process, from raw materials to finished products, in keeping with the goals of the Process Analytical Technology initiative.

Reference

1. *Encyclopedia of Rapid Microbiological Methods*; Miller, M.J., Ed.; Davis Healthcare International Publishing, Ltd.: River Grove, IL, 2005.

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