The Introduction of Qualitative Rapid Microbiological Methods for Drug-Product Testing

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FDA recently approved a set of rapid microbial methods for microbial-limit testing of drug products and pharmaceutical-grade waters—the first process analytical technology (PAT) validation under the agency's two-year-old initiative. Team members from FDA and GlaxoSmithKline report on the successful validation effort and identify points to consider for future projects.

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Conventional microbiological test methods, based on nineteenth-century techniques, are time-consuming and labor-intensive, lack sensitivity, and are subjective and poorly validated. Because microbiological testing is a bottleneck in product release, the pharmaceutical industry's interest in rapid microbiological methods (RMMs) has grown considerably during the past ten years.

RMMs such as adenosine triphosphate (ATP) bioluminescence and solid-phase laser cytometry are being investigated for the advantages they offer in speed, sensitivity, and accuracy. Despite significant progress in the development of such methods, however, the pharmaceutical industry has been hesitant to implement them for product testing because of concerns about regulatory acceptance and technical barriers. Technical and regulatory barriers include the complex chemical nature of the technology, the challenge of technical transfer of these systems from the food, beverage, and cosmetic industries (where they have been used for several decades) to the pharmaceutical sector, and the lack of appropriate guidance for validation and implementation.

Validation and regulatory requirements for RMM technologies are beginning to emerge, however, following FDA's endorsement of RMMs as a process analytical technology (PAT), as part of the agency's quality initiative, "Pharmaceutical CGMPs for the 21st Century: A Risk-Based Approach." PAT is a framework for scientific, risk-managed pharmaceutical development that requires timely, in-process measurement of materials and processes to ensure the quality of final products. Real-time or near-real-time microbiological testing can be conducted as part of manufacturing process control only through the use of RMMs.

GlaxoSmithKline (GSK) has taken the opportunity provided by the PAT initiative to establish rapid microbiological methods for microbial-limit testing of pharmaceutical-grade waters and product release of selected dosage forms. FDA's approval of these methods marks the agency's first approval of a PAT method. This article addresses some of the technical questions associated with introducing qualitative RMMs for drug-product testing, including aspects such as instrument qualification and microbiological performance testing.

Table I: Microbial-limit test validation criteria for rapid microbiological methods.

Parameter	Qualitative: ATP bioluminescence	Quantitative: laser cytometry
Accuracy		●
Precision		●
Specificity	●	●
Equivalence	●	●
Limit of detection	●	●
Limit of quantitation		●
Linearity		●
Range		●
Repeatability	●	●

Background

The current compendial methods for microbial testing of finished drug products or pharmaceutical-grade waters generally use either a membrane filtration-based or pourplate/spreadplate-based microbial-limit test (MLT). These growth-based test methods typically take four to seven days to complete and require visual examination and identification. The MLT defined by the current US Pharmacopeia is a threefold test, entailing

- enumeration of microorganisms (quantitative)
- determination of the presence or absence of pharmacopeial indicator microorganisms (qualitative), and
- identification of recovered microorganisms (qualitative).

Two new technologies for microbial determination, ATP bioluminescence and laser cytometry, are superior to traditional techniques in many respects, including sensitivity, selectivity, and speed. The ATP bioluminescence assay quickly and effectively determines the presence of viable microorganisms. Bioluminescence occurs naturally in the firefly (*Photinus pyralis)*, when the enzyme luciferase catalyzes the reaction of luciferin and the nucleotide ATP to produce light. By producing light, luciferase thus can quickly and accurately detect the presence of ATP (found in all living cells, including microbial cells). In laser cytometry, a fluorescent stain signals viable microbial cells. The stain is converted to a fluorescing state only when esterase enzyme activity and an intact cell membrane are present; nonviable cells thus are not detected.

Validation requirements for microbial-limit testing with RMMs are defined in PDA's Technical Report 33, "Evaluation, Validation and Implementation of New Microbiological Testing Methods" (1). The validation criteria, listed in Table I for both qualitative and quantitative methods, are independent of the technological platform. The equivalence of RMMs to conventional methods can be demonstrated through a series of experiments designed to assess the validation criteria, which can be performed by the prospective user or by others (*i.e.*, literature references can be used to address validation criteria; see the bibliography at the end of this article).

Validation of RMMs: a case study

GSK's microbiologists needed to determine the appropriate validation strategy and application of qualitative MLT methods for drug products and pharmaceutical-grade waters. Because

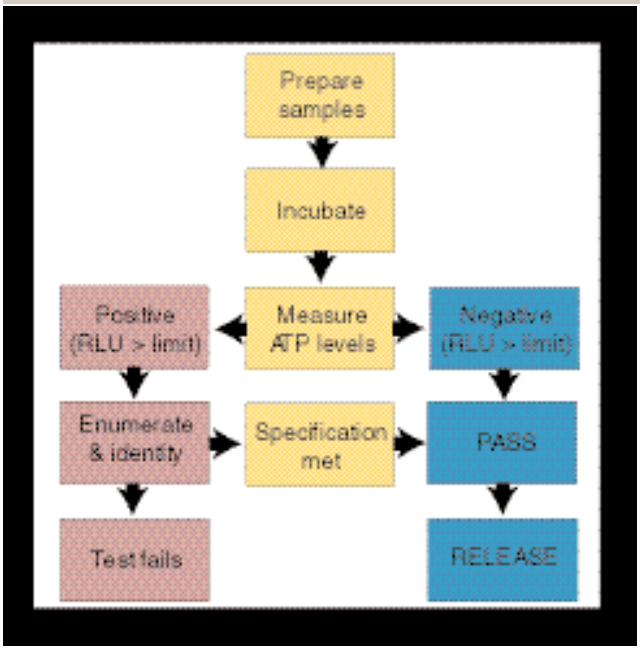

Figure 1: Qualitative testing strategy for two-tiered testing, using the ATP bioluminescence method and a conventional MLT method (RLU denotes relative light units).

of the diversity of requirements for product testing and monitoring, no single technology platform has the capacity to satisfy all testing requirements. Therefore, a two-stage method was selected, combining a rapid microbiological method for qualitative testing and conventional MLTs. In this approach, the RMM is used as a screening test; batches that pass need not undergo conventional testing. This type of screening is applicable for high-volume products where historical microbial data indicate that the product typically is free of bioburden. RMM screening allows rapid product release, in hours instead of days or weeks, for batches that demonstrate the absence of microorganisms.

The ATP bioluminescence method was validated as a microbial-limit testing screen, and laser cytometry was validated for quantitative microbial analysis of pharmaceutical-grade water. The enumeration and identification aspects of the products' acceptance criteria remained unchanged. Validation and implementation were no different for RMMs than for any other test, with the following exceptions:

- MLT acceptance criteria were revised for use with RMMs
- a strategy was developed for handling batches that gave positive results in the screening test.

Conventional acceptance criteria for microbial limits are not applicable to RMMs because RMMs are much more sensitive than conventional methods. Therefore, we adopted the following two-tiered specification:

- Tier 1 (RMM): negative (the result is below the set limit)
- Tier 2 (conventional method): The total aerobic microbial count is not greater than *x* colony-forming units per mL (cfu/mL), and S*taphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* species, *Escherichia coli*, and Enterobacteriaceae are absent in *x* mL **[QA: of sample?].**

Table II: Experiments for validation of the microbial-limit test using rapid microbiological methods.

Experiment	Acceptance criterion
Absence of interference (TLV)	The product formulation does not inhibit or enhance method performance, and a threshold limit value (TLV) has been established.
Specificity	The method has an acceptable ability to detect all the microorganisms with which it is challenged.
Limit of detection	The method has an acceptable ability to detect microorganisms in samples spiked with 1 to 10 cfu.
Ruggedness and repeatability	The method demonstrates acceptable ruggedness and repeatability in analyses with various analysts, various instruments, and various reagent batches.
Robustness	The method demonstrates acceptable robustness in analyses with deliberate variations of incubation time, shaking speed, incubation temperature, microbiological physiological conditions, and compositions of mixed cultures.

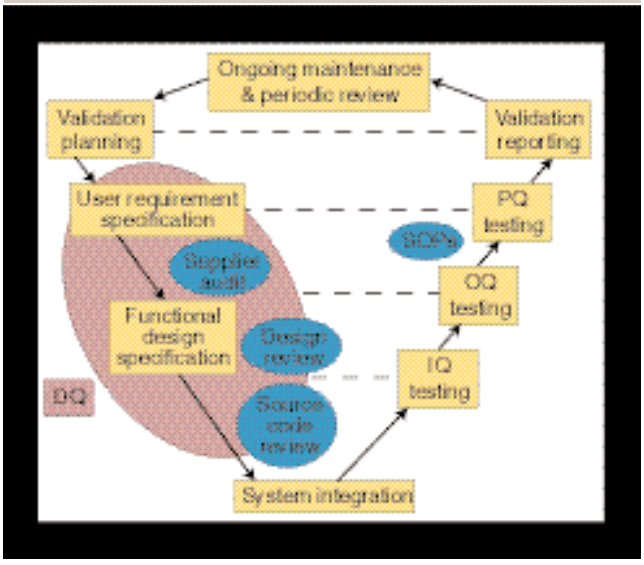

Figure 2: The V-model for system qualification.

A strategy was developed that allows for rapid release of bioburden-free products when the RMM yields negative results for the presence of ATP. When the screen indicates the presence of microbial ATP, the conventional MLT is performed to determine the numbers and types of microorganisms present and to demonstrate compliance or noncompliance with the microbiological specification. Figure 1 illustrates this process.

Validation of the RMMs focused on both microbiological testing performance and technology platform qualification. A validation model that GSK has used successfully with RMMs is a V-model, which is widely used in computer software development. The model was modified to include microbiological performance qualification attributes (see Figure 2). Other validation models exist, and the use of the modified V-model in this example is not meant to suggest that it is required. However, the basic elements of design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ), as illustrated in this model, are important parts of the validation of any new analytical method. The modified V-model also provides a convenient way to combine equipment qualification requirements with a microbiological performance evaluation.

Design qualification. The staff developed a validation master plan for RMM evaluation. The plan included both equipment and microbiological-performance evaluation details such as user requirements, performance capabilities, computer hardware and software requirements, supplier audits, costs, and benefits. The flow diagram shown in Figure 3 illustrates a process for assessing the feasibility of any rapid microbiological technology, using ATP bioluminescence as an example.

Installation qualification. The IQ stage verified and documented that the RMM instrumentation had been supplied, installed, and tested according to the manufacturer's specifications. The installation qualification package included documentation of a visual inspection of all equipment, copies of all operation manuals, and confirmation that all required utilities (*e.g.*, electricity, vacuum, and laminar flow hoods) were installed properly. Copies of protocols and results for all tests performed by the vendor and on site are maintained in the event of an inspection.

Operational qualification. The manufacturers of rapid microbiological technologies supply the protocols for the OQ of their instruments. These protocols may be used as is or modified by the end user as appropriate. Typical tests include verifying the interface between the software and the instrument, verifying user access to each input message or command processed by the software, cross-checking each external file or data record referenced by the supplier, and verifying output messages, displays, and recorded data generated by the software. These tests were performed and documented with both compendial microorganisms and site-specific environmental microorganisms. This OQ was critical at this stage, because it ensured correct operation of the method under working conditions.

Performance qualification. PQ demonstrated the suitability of the RMMs for the purpose of microbial-limit testing. Validation experiments were designed to demonstrate and justify the use of the RMMs for testing specific drug products and pharmaceutical-grade waters. PDA Technical Report 33 was followed in performing the validation experiments listed in Table II. Testing for each criterion (absence of interference, specificity, limit of detection, ruggedness and repeatability, and robustness) was outlined in protocols with specified acceptance criteria. The performance tests used for the ATP bioluminescence method are summarized below.

Absence of interference. Interaction of the drug product or sample matrix with the luciferase ATP enzymatic reaction (*i.e.*, inhibition or enhancement) was evaluated through tests of noncontaminated samples and samples spiked with the following contaminants:

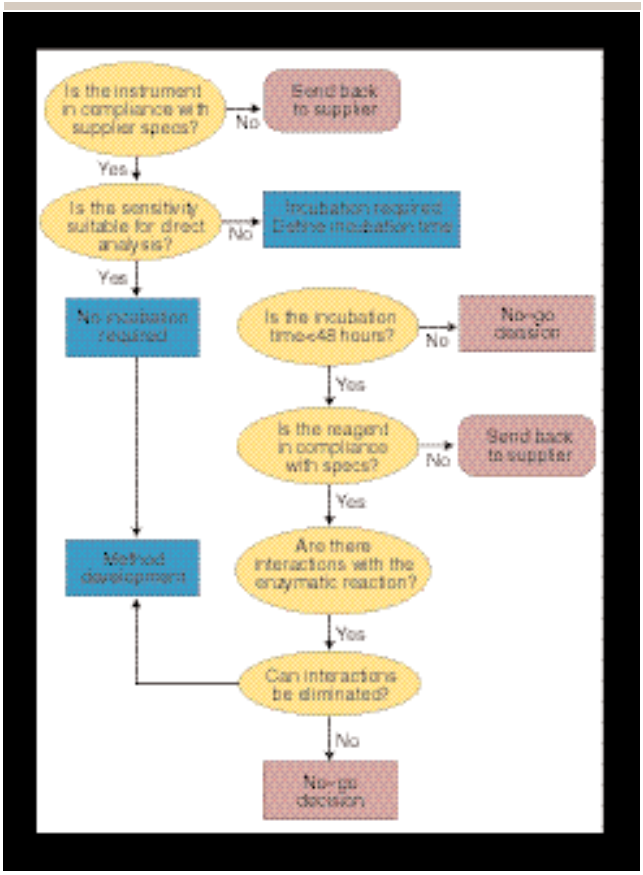

Figure 3: Process for design qualification of rapid microbiological technology, as applied to ATP bioluminescence.

- Gram-positive bacterial species: *S. aureus*
- Gram-negative bacterial species: *E. coli*
- Spore-forming bacterial species: *Bacillus subtilis*
- Fungus: *Aspergillus niger*

The results of these tests (multiple data points) were used to set the threshold limit value (TLV), above which samples are considered contaminated and below which they are considered not contaminated. The accepted probability of a false-negative result (identifying a contaminated sample as noncontaminated) was 0.00555%. This value is equal to the probability of a false-negative result accepted for the media fill test of sterile drug products.

Specificity. To demonstrate the test's ability to detect a range of microorganisms, at least six replicate samples of drug product were spiked with 10 to 100 cfu of microorganisms from site-specific isolates and the following compendial microorganisms (strains of the American Type Culture Collection, ATCC):

- *E. coli* (ATCC 8739)
- *S. aureus* (ATCC 6538)
- *P. aeruginosa* (ATCC 9027)
- *B. subtilis* (ATCC 6633)
- *Salmonella abony* (ATCC 6017)
- *Candida albicans* (ATCC 10231)
- *A. niger* (ATCC 16404)

The test's ability to differentiate between noncontaminated samples and samples containing diverse microorganisms was demonstrated by a two-way analysis of variance, using five contamination factors (threshold, fungus, and gram-negative, gram-positive, and spore-forming bacteria).

Limit of detection. The test's ability to detect low concentrations of microorganisms was demonstrated using samples of drug product spiked with 1 to 10 cfu of the same microorganisms used to test specificity.

Ruggedness and repeatability. The test's precision was demonstrated by analyzing the same bioburden-free samples under a variety of normal test conditions; two different analysts carried out the tests with two different instruments, reagent kit lots, and membrane lots, and results were compared.

Robustness. The test's ability to yield results unaffected by small but deliberate variations in method parameters was demonstrated by varying the following parameters:

- incubation time: from 0 to 24 h, using a gram-positive bacterial species and a fungus chosen for slow growth [were both chosen for slow growth or just the fungus?].
- shaking speed: 200 or 300 rpm, based on 250 rpm as established by current good manufacturing practices, using a gram-positive bacterial species and a fungus, [both?]chosen for high oxygen requirements.
- ∗ncubation temperature: 30°C or 35°C, using a gram-positive bacterial species, chosen as a representative bacterial species, and a fungus chosen for its temperature sensitivity.
- microbial physiological conditions: soybean-casein digest medium at pH levels of 7.3, 5.5, or 3 and a sodium chloride concentration of 0.5, 1.5, or 5%, using gram-positive and gram-negative bacteria. The conditions were chosen to challenge the test system, because the bioluminescence enzyme complex is sensitive to high and low pH as well as to high salt concentration.
- mixed cultures: (a) gram-positive and gram-negative bacteria and a fungus or (b) a spore-forming bacterium and a fungus.

Equivalence. The criterion for equivalence is that the new method be at least as good as the current method. In conventional MLT methods, the limit of detection is approximately 10 cfu/mL, whereas RMMs can easily detect microbes at the single-cell level. Taking into account this significant increase in sensitivity, a multifaceted approach was used to establish method equivalence, combining a review of the published literature, laboratory experiments with spiked samples, and parallel testing of pharmaceutical-grade water samples with rapid and conventional methods. In the case of the ATP bioluminescence method, literature citations were sufficient to establish equivalence. Figure 4 shows an example of the results obtained in parallel testing of pharmaceutical-grade water with a pourplate method and with laser cytometry over a 20-day period. The results show similar trends; however, the conventional methods took several days, while the laser cytometry results were obtained within hours, without the need for pre-enrichment.

Moving from data to knowledge

Broad acceptance and implementation of rapid microbiological methods by the pharmaceutical industry will be a slow process. Given the range of skills required, equipment qualifi-

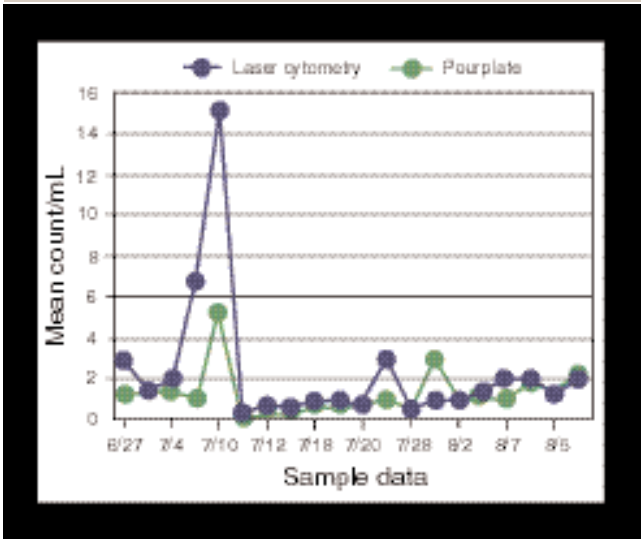

Figure 4: MLT results for purified water tested by a conventional pourplate method and by laser cytometry.

cation and microbiological performance validation are best tackled through a team approach involving the system manufacturer to ensure that expertise is effectively transferred from the manufacturer to the operators using the equipment.

It is essential that during the various stages of evaluation, implementation, and validation, a thorough understanding of the new testing system and a true picture of its capabilities be gained. Data generated must be translated into knowledge, which must be effectively documented through a comprehensive validation process and passed on through a training program.

Future direction

Interest in rapid microbiological methods in the pharmaceutical industry is high and is expected to increase following reports of successful implementation. Adoption of RMMs is warranted by significant advantages in speed of results, process efficiency savings, sensitivity, and business benefits.

The advent of PAT has given a new impetus to the introduction of RMMs. Conventional microbiological test methods are not capable of delivering real-time or near-real-time results, a prerequisite for successful exploitation of PAT benefits. Because RMMs do have this capability, they will be an invaluable aid to successfully realizing the objectives of PAT—increased quality and efficiency.

The current regulatory environment for implementing rapid microbiological methods also is encouraging. The regulatory authorities in the United States and Europe have approved [these?] new PAT technologies for nonsterile product release testing and pharmaceutical-grade water testing, and it is likely they will approve PAT applications for sterile products and continuous environmental monitoring in the near future.

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