Considerations in the Evaluation of Rapid Microbiological Methods

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What are Rapid Microbiological Methods (RMM)

During the past 20+ years the field of Rapid Microbiological Methods (RMM) has gained momentum. RMM provide results that are equivalent in performance or better and significantly reduce the time to results. They are based on technologies which can be growth-based, labeled cell based, or surrogate-based cellular markers for a microorganism (i.e., nucleic acid-based, fatty acid-based). RMM are frequently automated, and possess increased sensitivity in detecting changes in the sample matrix (e.g., by-products of microbial metabolism), under conditions that favor the growth of microorganisms. This presentation will focus on growth based methods.

Many rapid microbiological methods provide more sensitive, accurate, precise, and reproducible test results when compared with conventional methods. Furthermore, they may be fully automated, offer increased sample throughput, operate in a continuous data-collecting mode, provide significantly reduced time-to-result (e.g., from days or weeks to hours or minutes).

Current RMM can detect the presence of diverse types of microorganisms or a specific microbial species, enumerate the number of microorganisms present in a sample, and can identify microbial cultures. The manner in which microorganisms are detected, quantified or identified will be dependent on the specific technology and instrumentation employed. For example, growth-based technologies rely on the measurement of physiological markers that reflect the growth of microorganisms. These types of RMM require the organisms from the test sample to proliferate in order to be detected and/or be quantified. Growth based rapid methods continue to use conventional liquid or agar media, that are similar to traditional methods making their validation simpler.

Examples of the types of core technology principles that are currently used in growth-based RMM are based on impedance microbiology, optical system utilizing color or fluorescence detection of carbon dioxide (CO_2), the utilization of biochemical and carbohydrate substrates, the use of digital imaging and auto-fluorescence for the rapid detection and counting of micro-colonies, fluorescent staining and enumeration of micro-colonies by laser excitation, cell staining followed by Flow Cytometry, and detection of microbial contamination using ATP bioluminescence.

Benefits of RMM

Savings from faster detection an early intervention- Faster detection of contamination results in early intervention and faster corrective action. This will result in faster substitution of out of spec raw materials and faster resumption of production of high quality product. Faster detection of problems can result in less production of defective products. A single incident of faster intervention can save more than the cost of a new rapid detection system.

Lower cost of inventory- Speeding up microbiology testing will eliminate idol time thereby speeding up production and reducing inventory. Significantly decreasing the manufacturing cycle, result in the lowering of the required working capital investment, and faster release of inventory.

Increased capacity- By implementing a rapid microbiological method, floor space can be reallocated to increase manufacturing capacity, and add more SKU's to inventory, or reduce distribution center costs.

Decrease cost of capital- If batches or products are stored waiting test results, the RMM will benefit by reducing the

working capital investment in inventory, and reducing inventory, will clear out space in the warehouse.

Improvement in account receivable- RMM will allow for faster order fulfillment by reducing the time it takes to produce, and test products. Reducing the time required to complete orders leads to faster invoicing and faster payment.

Improved Risk Management- At the time of a contamination event, when so much is at risk, the benefits of rapid methods are increased. The faster a problem is identified, the more quickly corrective action and recover can be initiated. RMM reduce the potential negative impact and minimize risk for the bottom line.

Rapid methods tend to carry higher costs than conventional methods, but may also deliver significant savings in the longer term. The operational and financial benefits far outweigh the expense. When combining all of the benefits listed above the cost of RMM per assay is far less than that of traditional methods.

Criteria for the Selection of RMM

Accuracy of Results: The degree of accuracy and specificity, minimal operator variability, ease of use, low false positive rate, and effectiveness with a range of sample configurations are important parameter in choosing an RMM.

Timeliness and automation of Results: Rapid results allow for early intervention, reduces risk, decreased inventory, and other financial benefits. However, the results must be actionable and must be compatible with the laboratory work day. Automation of data achieving and automatic transfer of information to where it is needed can be essential parameters for a RMM.

Depth of platform: the range of raw materials, in process and finished products that can be analyzed as well as the breath of assays that can be performed on the platform. The full economical benefits of the RMM can only be realized if the vast majority of the company products can be analyzed with the RMM and if all, or at least most, of the required assays can be performed on the RMM platform.

System capacity: How many samples can be processed by the system on a daily basis, its ease of use and the training level of the system operators. The system capacity needs to fit ones requirement for testing. The system must be able to process all the required samples. The labor involved in sample preparation, sample monitoring and results analysis as well as the system ability to minimize operators errors.

System Support: The commitment of the systems vendor to support the system and provide timely answers to questions is another consideration.

Validation Requirements

Method validation is the process used to confirm that an analytical procedure employed for a specific test is reliable, reproducible and suitable for its intended purpose. All analytical methods need to be validated prior to their introduction into routine use, and this is especially true for novel technology platforms, such as RMM. USP <1223> (and its equivalent Eur. Pharm. 5.1.6) as well as ISO 16140 supply guidelines and protocol for the validation of alternative methods in microbiology. The table below show some of the criteria used:

Table 1: Validation Characteristics for an RMM Validation

Validation Characteristic	Definition
Limit of Detection	The lowest number of microorganisms detectable in the sample matrix. The limit of detection is determined by inoculating samples with serial dilutions of viable contaminants, with Colony Forming Units (CFUs) confirmed at inoculation by plate counts. Challenge testing is performed with each challenge microorganism using an amount less than 100 CFU but greater than 10 CFU. Any growth is recovered and identified to confirm identity.
Specificity	The ability of the test method to detect a panel of organisms within the sample matrix established in the validation protocol. Specificity is demonstrated by challenging the RMM to detect variations in microbial growth characteristics and concentrations.
Precision	Precision is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of suspensions of microorganisms across the range of the test. Precision is usually expressed as the variance, standard deviation or coefficient of variation (CV) of a series of measurements.
Ruggedness	The degree of reproducibility of results obtained by analysis of the same sample under a variety of normal test conditions, such as different analysts, different instruments, and different reagent lots. Ruggedness is the lack of influence on the test results of operational and environmental variables of the microbiological method and should be assessed by having different analysts prepare multiple samples as specified in procedures.
Robustness	The capacity of a method to remain unaffected by small, but deliberate, variations in method parameters (e.g., changes in reagent concentration or incubation temperatures).
Comparative testing to Standard method	A side-by-side method comparison study consists of parallel testing of the new method alongside a standard-approved reference method to determine whether the performance of the new or modified method is acceptable compared to the reference method.

A key to the success of RMM is a robust validation process. Prior to method validation, it is critical to implement an Installation Qualification (IQ) Operational Qualification (OQ) Performance Qualification (PQ) of the equipment used the software and the databases. IQ verifies and documents that the system was installed as specified in the appropriate laboratory environment. OQ verifies and documents the instrument and method work for the designated tests. PQ verifies and documents the performance with environmental organisms and compendial organisms for routine testing of batches of product.

The new Technical Report No. 33

Technical Report No. 33, Revision 2013 (TR 33):Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods was intended to provide guidance for the successful evaluation, validation, and implementation of alternative and rapid microbiological methods needed by the pharmaceutical, biotechnology and medical device industries to assure product quality. This technical report was written to establish industry-wide criteria on what constitutes an acceptable alternative or rapid microbiology test to the compendial or classical method and how to prove it to the satisfaction of quality organizations and regulatory agencies.

The technical report provides a practical guidance for "how to" validate new microbiology methods and associated systems and instrumentation. Specific guidance including acceptance criteria to be used when evaluating a new method for accuracy, precision, specificity, limit of detection and quantification, linearity, range, ruggedness, robustness and equivalence. Additional considerations with regard to software validation, system noise, false positives, false negatives and the use of environmental isolates are provided.

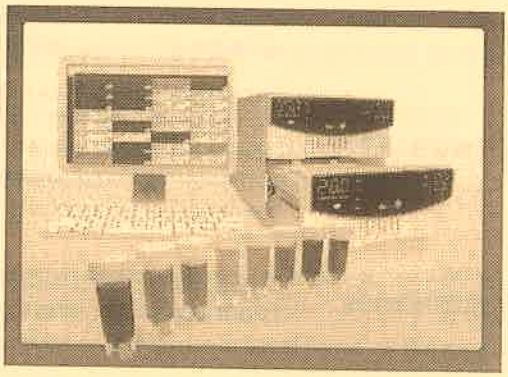
Choosing the best statistical strategy is one of the most challenging aspects of qualifying a new microbiology method, especially when the new method will take the place of an existing method. A section of TR 33 is devoted to demystify the use of statistics and satisfy the guidance expressed in both the USP and the Eur. Ph. informational chapters on the same subject. For example, TR33 provides direction and practical examples on how to choose the appropriate statistical model based on sample size, expected recovered counts and confidence levels. There is a new section on method suitability provides enhanced guidance on false positive and false negative testing. The purpose of suitability testing is to demonstrate that the new method will be compatible with specific product or sample matrices that will be routinely assayed.

Case Study – The BioLumix System

Note: a more comprehensive description of this case study can be found in Miller (2012) and Eden and Brideau (2013).

The system

The system is based on monitoring changes in a broth medium with unique dyes in which target microorganisms grow and are detected by changes in color or fluorescence. Test vials utilize a dual-zone configuration, where the sample is added to the upper, incubation zone, and an optical sensor monitors changes in color or fluorescence within the lower, reading zone. The upper and lower zones are physically separated from each other, thereby eliminating masking of the optical pathway by sample debris or by turbidity arising from dividing microorganisms. The temperature controlled instrument is capable of monitoring 32 samples simultaneously, collecting optical data 10 times an hour. Up to 32 instruments can be connected to one instrument. The software is validated to meet 21 CFR Part 11 (FDA, 2007) requirements, provides an audit trail, operator log in and log out, and provides various data reports. Detection events are automatically displayed. Results can be communicated over any IP network in real-time. Reports can be customized to fit the user's needs. The system supports bar code entry of tests. Data is evaluated in real time to enable the product's release in a timely manner.



The Total Aerobic Count Vial and the Total Yeast and Mold Vial utilize a CO_2 sensor located at the bottom of the vial, where a general medium is used in the Total Aerobic Count Vial, and a selective medium with added antibiotic (to suppress the growth of bacteria) is used in the Yeast and Mold Vial. The Enterobacteriaceae Vial monitors changes due to a pH shift as Enterobacteriaceae organisms (Gram negative, bile tolerant) ferment glucose in the presence of a selective medium. The *E. coli* Vial monitors the utilization of MUG (4-Methylumbelliferyl-3-D-Glucuronide). The *Staphylococcus* Vial monitors changes due to a pH shift as *Staphylococcus* utilizes mannitol in another selective medium. Both the *Pseudomonas* and *Salmonella* Vials use the same CO_2 sensor platform as in the Total Aerobic Count Vial, but each utilize a specific selective medium for the growth of each organism.

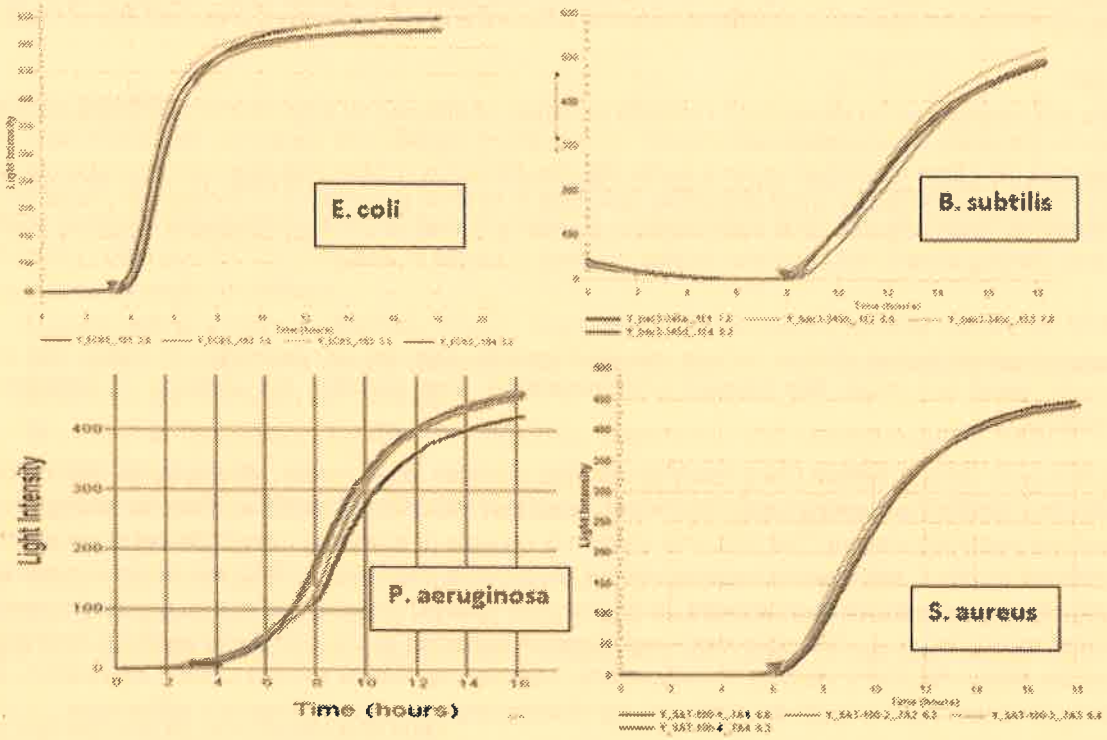
Dilute to spec may be used to estimate the number of organisms in the original test sample by first diluting the sample to the specification limit required for in-process or finished product release. The diluted sample is then introduced into the relevant test vial. If there is microbial growth in the vial, the number of organisms in the sample is higher than the required specification level; if there is no growth in the vial, the number of organisms in the original sample is lower than the required specification level. Although this is a semi-quantitative method, this procedure is appropriate for determining if a test sample is above or below a threshold level that is directly correlated with a quantitative specification, action or alert level. This is similar to using liquid medium in a Most Probable Number, or MPN procedure.

Side-by-side comparison

Naturally contaminated samples as well as inoculated products were used for each assay, in a number of studies. Several hundred samples of non-sterile over-the-counter (OTC) medicine such as vitamins, antacids, suppositories, laxatives, ibuprofen, aspirin, etc. as well as cosmetic and toiletry products were tested for each assay. Some of the samples were inoculated with target organisms. Agreement between the BioLumix system and the USP methodology was 100% in many cases and always above 98%.

Precision

Precision is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of suspensions of microorganisms across the range of the test. Precision is usually expressed as the variance, standard deviation or coefficient of variation (CV) of a series of measurements. Generally, a %CV of 15-35% is acceptable. For the purpose of demonstrating precision in this RMM, a series of replicate tests were performed using different concentration levels and a variety of challenge microorganisms. An example of the results is shown in the figure. The data from these studies show that the degree of agreement among individual test results, for the BioLumix method for suspensions of more than 50 different species and strains of a wide variety of microorganisms, is well within the acceptable range for a suitable level of precision.



Specificity

It is necessary to demonstrate, for each test vial, that the target organisms are, in fact, detected, while other non-target organisms are not detected. This is also referred to as inclusivity and exclusivity. Target organisms that should be detected within the test vial were inoculated at levels ranging from 10 to 1,000 CFU/vial, and non-target organisms were inoculated at levels ranging from 10,000 to 100,000 CFU/vial.

For Total Aerobic count 125 diverse species of bacteria were separately inoculated into vials. All were detected in the vials (no non-target testing was conducted, as the growth medium in this vial is universal). For yeast and mold, 42 different strains were evaluated in the vial. All of the yeast and mold were detected. 25 species of various bacteria were evaluated and none were detected in the vial. 52 strains of Enterobacteriaceae family were inoculated into the Enterobacteriaceae Vial, all of these organisms were detected; 23 Gram- negative and 28 Gram-positive species of bacteria that did not belong to the family Enterobacteriaceae none of these non-target organisms were detected. Similar results were obtained for the *E. coli*, *Staphylococcus, Pseudomonas* and *Salmonella*. Therefore, inclusivity for the target organisms and exclusivity for the non-target organisms was demonstrated.

Sample	BioLumix DT (hours)	Plates cfu/g
1	10.2	6
2	10.4	3
3	10.6	3
4	10.6	2
5	10.6	2
6	10.8	1
7	11.0	0
8	11.2	0
9	ND	0
10	ND	0

Average count of sample 1.7 cfu/g.

Limit of detection

Limit of Detection (LOD) is the lowest number of microorganisms in a sample that can be detected under the stated experimental conditions. Various cultures of microorganisms were decimally diluted to achieve low levels of inoculation into growth media or samples. The goal was to achieve a level of 1-10 CFU/mL. One mL of the solution was added to

several vials and the same amount was added to a plate. Limit of detection was calculated for each one of the assays (total count, yeast and mold, Enterobacteriaceae, etc.) . The example shows the results for Total Aerobic count with 10 replicates of a sample containing ~ 1.7 cfu/g. The collected data showed that the LOD for the BioLumix System were equivalent to the plate method in detecting low numbers of organisms as directly compared to the plate count method.

Ruggedness

Ruggedness is the degree of precision of test results obtained by analysis of the same samples under a variety of normal test conditions, such as different analysts, instruments or reagent lots. The BioLumix System has been demonstrated to be very rugged with high precision of test results obtained by analyzing the same microorganisms under a variety of different operating conditions including different analyst, instrument units, and lots of media.

Robustness

Robustness is a measure of the capacity of a method to remain unaffected by small deliberate variations in method parameters. In this study 3 parameters were tested: (i) Instrument temperature variations; (ii) Sample size introduced into the vial; and (iii) Effect of medium volume in the vial. the BioLumix System has been demonstrated to be very robust and remains unaffected by small deliberate variations in method parameters. It shows high precision of test results obtained by analyzing the same microorganisms under a variety of different conditions including varying temperatures, varying sample volumes and varying volumes of media in vials.

Summary

The automated growth-based system detects microbial growth, provides an estimation of viable cell counts (total aerobic count, yeast and mold, bile tolerant Gram-negatives), and identifies the absence of specified groups of microorganisms.

The novel BioLumix Optical System using ready-to-use vials provides faster results, labor savings, automation, and connectivity. The streamlined testing design and rapid, accurate results lead to reduced material-holding time for faster product and raw materials release. The BioLumix System is capable of analyzing many different types of products including colored samples, and viscous material without any product interference. BioLumix offers a comprehensive range of microbiological tests for raw materials, in-process and finished products, as well as processing water. Environmental monitoring (e.g., detecting micro-organisms on surfaces) is also performed easily by inserting the swab directly into the assay vial. Early warning of contaminated samples, as well as sample release information, could be automatically communicated through your intranet, significantly improving your company's efficiencies.