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USING ATP BIOLUMINESCENCE FOR MICROBIOLOGICAL MEASUREMENTS IN PHARMACEUTICAL MANUFACTURING

Lucia Ceresa

Pall Life Sciences
Milano, Italy

and Peter Ball

Pall Life Sciences
Portsmouth, Hants, UK

INTRODUCTION

The microbiologist in a pharmaceutical company who is faced with the decision regarding which rapid microbiology system to purchase is faced with a very broad choice of products varying significantly in cost and technical complexity. Documents such as the Parenteral Drug Association (PDA) Technical Report 33 *Evaluation, Validation and Implementation of New Microbiological Testing Methods* (PDA 2000) focus on the technical aspects of making this decision (some key aspects of this are covered later in this chapter). However, just as critical to the successful routine implementation of a rapid microbiology system are economic

factors, particularly return on investment, regulatory factors such as ease and speed of validation, together with practical factors such as the ability to use the chosen technology across a broad range of applications, the complexity and length of training required and ease of use.

Adenosine triphosphate (ATP) bioluminescence is a well-established technology and has already gained regulatory acceptance as a tool for releasing certain types of pharmaceutical products. This chapter focuses on this technology and covers practical aspects of introducing ATP bioluminescence into Pharmaceutical Microbiology Quality Testing using the Pallchek™ Rapid Microbiology System as an example.

GUIDANCE DOCUMENTS ON ADOPTING RAPID MICROBIOLOGY METHODS

There are several important guidance documents relevant to the adoption of rapid microbiology by a pharmaceutical industry user. These will not be discussed in detail here, although reference will be made to key parts of these documents where relevant later in this chapter. The reader is strongly recommended to review these documents as they contain important information that can assist in the process of selecting and validating new rapid technology. The three key documents are:

- PDA Technical Report 33 (PDA 2000)
- United States Pharmacopoeia draft general information chapter 1223 (Pharmacopoeial Forum 2005)
- European Pharmacopoeia draft chapter 5.1.6 (Pharmeuropa 2004)

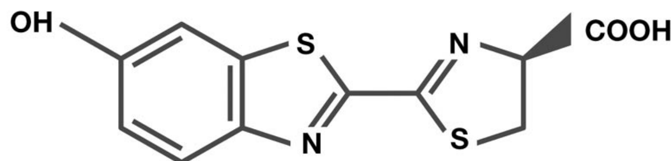
DETECTION OF MICROBIAL CONTAMINATION USING ATP BIOLUMINESCENCE

Adenosine triphosphate (ATP) is a ubiquitous molecule that acts as a major energy storage medium for all living cells, including microorganisms. ATP consists of a ribose group with an adenine moiety on one side of the molecule and three phosphate groups on the other. Hydrolysis of these phosphate groups releases energy and drives a wide variety of biological reactions within the cell. The ATP molecule can thus be used as a marker of cellular contamination.

Measurement of ATP using methods typically based on the luciferin-luciferase enzyme system (see below) has been used for many years in a wide variety of applications, including contamination monitoring in industries such as food and beverage production. In the case of pharmaceutical manufacturing, where many raw materials, processes, and finished products typically contain very low levels of ATP, measurement of ATP contamination provides a good indicator of microbial contamination. Because the ATP content of microbial cells is relatively consistent, there is a correlation between ATP level and contamination level.

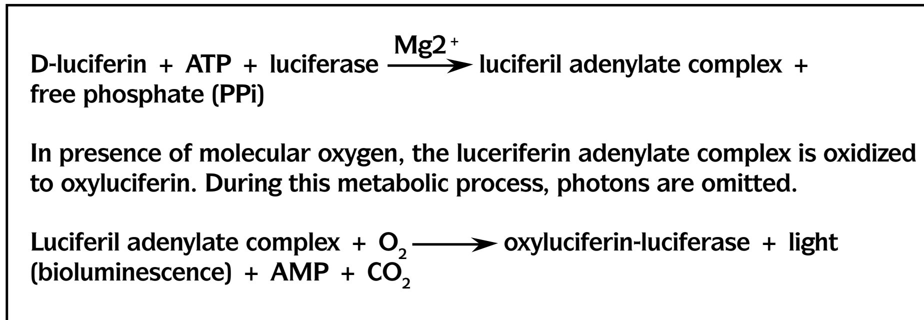
The most common method for detecting ATP based on bioluminescence measurement is using the luciferin substrate/luciferase enzyme system, which utilises material extracted from the firefly, *Photinus pyralis*. Firefly luciferase (luciferin 4-monooxygenase, EC 1.13.12.7) is a 62 kilodalton molecular weight protein. A detailed structure is given in Conti et al. (1996). In outline, the protein is folded into two compact domains. The large N-terminal domain consists of a beta-barrel and two beta-sheets. These beta-sheets are flanked by alpha-helices to form an alpha beta alpha beta alpha five-layered structure. The C-terminal portion of the molecule forms a distinct domain, which is separated from the N-terminal domain by a wide cleft. This cleft contains the active site that metabolises the substrate, luciferin. Firefly luciferin ((S)-4,5-dihydro-2-(6-hydroxy-2-benzo-thiazoloyl)-4-thiazolecarboxylic acid) is a dioxetane-like molecule (White et al. 1961) that is the substrate for the luciferase enzyme (see Figure 1).

Figure 1. Structure of the Unoxidised Form of Luciferin from *Photinus pyralis.* The Molecule has a Dioxetane-like Structure.



ATP measurement based on bioluminescence using the luciferin-luciferase substrate-enzyme complex relies on oxidation of luciferin by the luciferase enzyme (see Figure 2).

Figure 2. The reaction sequence for ATP bioluminescence based on luciferin-luciferase.



This reaction requires ATP, oxygen and magnesium ions. Luciferin oxidation results in photon emission with maximum energy (λ max) at 562 nm. The reaction is pH and temperature-dependent. For ATP bioluminescence measurements, photons are normally detected and quantified using a sensitive photomultiplier tube or CCD device. Typically, the count of photons per second detected is reported as Relative Light Units (RLU).

The detection sensitivity of ATP bioluminescence technology depends on a number of factors, discussed in more detail later. Principle factors are:

- **Background light level**. In addition to environmental sources of light, photons can arise from items such as plastic disposables (which are frequently sterilised using high-energy sources such as gamma irradiation).
- **Sample composition**. As mentioned above, the λ max for the luciferin-luciferase reaction is pH sensitive, with optimal pH of 7.75. Under acidic conditions, the λ max shifts from 562 nm (yellow-green light) to longer wavelengths (red) and under basic conditions, light output is significantly reduced. Other potential sample interference effects include enhancement of signal, high intrinsic background (either due to ATP contamination and/or other sources of photons unrelated to ATP), and signal quenching.

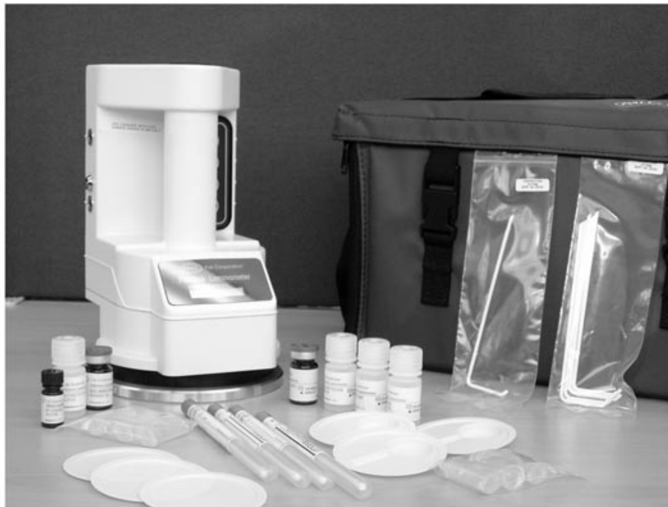
Practical guidelines for identifying and minimising these potential sources of interference are given later.

THE PALLCHEK RAPID MICROBIOLOGY SYSTEM

As mentioned previously, this chapter covers practical aspects of the adoption and validation of rapid microbiology technology using the **Pallchek** Rapid Microbiology System as a working example.

The Pallchek Rapid Microbiology System consists of a compact and portable luminometer and reagent kits (see Figure 3).

Figure 3. The Pallchek Rapid Microbiology System.



The system is designed for the detection of microbial contamination based on measurement of ATP using the luciferin-luciferase substrate/enzyme system from the firefly, *Photinus pyralis*, as described above. Unlike many systems that use ATP bioluminescence, in which light output occurs as a flash (with a duration typically measured in milliseconds), the reagent system utilized by the Pallchek Rapid Microbiology System results in the emission of photons over several minutes. Because of this characteristic, simple manual addition of reagents followed by measurement using a hand-held luminometer provides accurate and reproducible data. This is in contrast to many other methods based on ATP bioluminescence, which require addition of reagents by the instrument making measurements. This has two practical consequences: firstly, the timing from reagent addition to measurement using this system is relatively non-critical and secondly, the Pallchek luminometer is simple in design and hence lower in cost.

The Pallchek Rapid Microbiology System is designed for maximum versatility of operation. It can be used to perform direct measurement of microbial contamination on a membrane, in liquid samples, and on surfaces, as well as measurement of surface contamination using swabs.

For maximum sensitivity, measurements are usually made on liquid samples that have been collected using a membrane filter. After filtration of the sample, the membrane filter used for the analysis can be washed to remove any components present in the sample that might interfere with ATP-based bioluminescence measurement. The reagents are then added directly on to the surface of the membrane using micropipettes fitted with disposable plastic tips certified by the supplier as low in ATP. Direct contact between reagent and any microorganism concentrated on the surface of the membrane filter allows an enhancement of the sensitivity of ATP detection. This enables very reproducible results to be obtained. For critical applications, where very low levels of microbial contamination are expected, following sample collection and processing, the membrane can be incubated in liquid growth medium (an enrichment step). This enrichment step allows detection down to one colony forming unit (CFU) for a broad range of microorganisms.

Two different reagent kits are supplied by Pall to match the detection sensitivity required by the user. The maximum sensitivity achievable using the High Sensitivity Bioluminescent Reagent Kit (PN 7142) is 10^{-17}moles of ATP and when using the Standard Sensitivity Bioluminescent reagent (PN 7141), the maximum sensitivity is 10^{-16} moles of ATP.

The reagent kits supplied for use with the Pallchek Rapid Microbiology System contain three key components:

- An **extractant solution,** which solubilises the lipid-containing cell wall components of microbial cells and thus lyses these cells releasing the intracellular ATP.
- A lyophilised **luciferin-luciferase** reagent.
- A **reconstitution buffer**, for reconstituting the lyophilised luciferin-luciferase reagent. This solution maintains the sample at neutral pH, which, as stated above, is critical for optimal light output, and also supplies magnesium ions, which (as mentioned previously) are an important co-factor in the luciferin-luciferase reaction.

In addition, low ATP micro-centrifuge tubes are supplied to allow the luciferin-luciferase reagent to be dispensed and stored if it is not intended to use the entire reagent volume immediately following reconstitution.

Analysis of a sample using the Pallchek Rapid Microbiology System involves three key steps:

- Apply extractant solution to the sample. Wait approximately 15 seconds.
- Apply luciferin-luciferase reagent.
- Initiate a reading with the **Pallchek** luminometer within 5 seconds.

The sample being analysed is located in a disposable, low ATP, plastic sample tray, which is placed in an aluminium test plate. This aluminium test plate provides an easily cleaned, rigid, stable surface against which the **Pallchek** luminometer can form a light-tight seal (see below).

The Pallchek luminometer is operated by pushbuttons located on the front of the instrument adjacent to the handle (see Figure 3). When the luminometer is switched on and ready for use, the liquid crystal display displays the message **<READY TO COUNT**>. To take a reading, the luminometer is placed centrally over the aluminium test plate containing the sample. The luminometer is then pressed downwards. This action activates a vacuum pump within the instrument, which creates a vacuum under the silicone ring fitted to the base of the instrument. Creating a vacuum between the silicone ring and the aluminium test plate ensures a light-tight seal and minimises extraneous light from interfering with readings. During this phase of testing, the instrument displays the message **<PUMPING>**.

Once a satisfactory vacuum has been created, the luminometer checks for the absence of extraneous light and measures the background signal from the photomultiplier tube. During this phase of testing, the instrument displays the message **<CHECKING>**. The instrument will then display the message **<COUNTING>**, during which time the instrument takes 10 consecutive measurements. At the end of this phase, the luminometer displays the average of these 10 measurements and prints the data, measured as RLUs using a separate printer.

The overall test cycle takes about 20 seconds.

CONTROLLING BACKGROUND

The key to achieving reproducible and sensitive results when using ATP bioluminescence technology to measure microbial contamination is controlling the background (i.e., sources of photons unrelated to the presence of ATP from

microorganisms). The main two sources of background are extraneous light and free ATP (i.e., any extracellular ATP not attributed to the presence of microbial contamination).

As mentioned previously, many materials pick up energy from exposure to radiation, including visible light and will re-emit this adsorbed light energy. This light can be detected by the **Pallchek** luminometer. For this reason, testing areas and materials should be protected from direct light to help prevent this phenomenon. Working under a laminar flow hood with the lights off (including U.V. light), will help to reduce or eliminate background due to light emission and will also prevent potential luciferase decay. Similarly, the user should avoid the use of plastic disposables that have not been characterized for low background, since sterilization of plastics using gamma and electron beam radiation can also be a source of background.

The second main source of background is ATP that may be present in the sample from sources other than any microbial contamination that is being measured. Both eukaryotic and prokaryotic cells contain ATP, and this ATP leaks from the cell following cell death. Autoclaving does not eliminate ATP. All materials to be used for ATP bioluminescence detection should be ATP-free or should have been demonstrated to have a low and stable ATP content.

For the **Pallchek** Rapid Microbiology System, the recommended analysis method is direct measurement of a filtered liquid sample. In this method, the sample is filtered through an appropriate analysis membrane, which is then flushed with an appropriate solution and any microbial ATP and then measured. Filtration and thorough washing of the analysis membrane will minimise any potential interference between the sample and microbial ATP in cells captured on the membrane, including potential interference from free ATP. Use of this method helps assure stable and controlled background measurements. The stability and reproducibility of background is considered critical to validation and regulatory acceptance of this type of method and that is why this is the recommended procedure for using the **Pallchek** Rapid Microbiology System.

PRACTICAL ASPECTS OF ROUTINE USAGE

There are a number of simple, quick steps that should be observed to ensure consistent results during routine testing:

Handling and Storage of Reagent Kits

Typically, luciferin-luciferase reagents need to be stored at low temperature. Follow the manufacturer's recommendations.

Good Laboratory Practice During Testing

In order to ensure consistent results, careful attention must be paid to the following practical aspects of using the **Pallchek** Rapid Microbiology System:

- As with any microbiological method, the use of aseptic techniques during reconstitution and dispensing of reagents is essential.
- Always wear powder-free gloves to prevent potential ATP contamination.
- ATP is present in the environment from a number of sources (including operators performing the test) and this can affect results. Perform testing in the same type of environment as other critical microbiological testing (e.g., sterility testing) and always use aseptic techniques for handling samples.
- *Sterile* does not mean ATP free. Autoclaving and other methods of steam sterilization do not eliminate ATP. This is particularly important to recognize when reusable equipment (e.g., membrane filter holders) is being used. For this reason, the use of sterile disposable equipment is preferred.
- All materials used for ATP bioluminescence measurement must be ATP-free or should have been demonstrated to have a low and stable ATP content. This includes items such as disposable plastic pipette tips that are supplied as *sterile*. Generally, all materials that are endotoxin free or certified as "ATP free" are suitable for use.
- Prior to testing samples, background readings of a sterile sample and suitability testing for the instrument and reagent controls must be performed as described below.
- Clean work surfaces at the end of each day using sterile wipes and isopropyl alcohol (IPA). If surfaces are cleaned with pure alcohol, rinse the cleaned surface thoroughly with sterile deionized water.

- For the Pallchek Rapid Microbiology System, the aluminum test plate should be cleaned with IPA at the end of each day. If alcohol is used to clean the test plate, it should be thoroughly rinsed/wiped with sterile, de-ionized water prior to resuming testing. Do not autoclave because autoclaving does not remove ATP.
- Usually, cleaning must be done the night before testing and the use of sterile wipes is also recommended. Taking background measurements (aluminum plate, preliminary suitability testing or surface) immediately after cleaning with a disinfectant will affect the results.
- Not all the cleaning agents are compatible with the ATP bioluminescence method. In particular, chemicals that react adversely with proteins can potentially interfere with the enzyme. In particular, acetone and benzalkonium chloride should be excluded from the testing area.

APPLICATIONS

Early Release of Pharmaceutical Products

As stated previously, one of the factors that can assist the prospective user of a rapid microbiology system to justify purchase of new technology is a strong economic argument based on a quick return on investment (ROI). For many users, the strongest economic argument for adopting rapid microbiology is early release of finished product.

The exact saving achievable by earlier release of finished pharmaceutical products obviously varies depending on a number of factors, but personal communication from existing users as well as some reports at global meetings on rapid microbiology indicate typical savings of the order of $100,000 per product per year for a product that is normally released at 5-7 days with the compendial method for the Microbial Limit Test instead being released at 24 hours with the Pallchek Rapid Microbiology System.

A key application for the Pallchek Rapid Microbiology System in product release is early release of so-called non-sterile products. A non-sterile product is one that is not claimed as sterile but which is required to have a very low bioburden. Examples of this type of product include nasal sprays and topicals. Personal communication from manufacturers of these types of products is that,

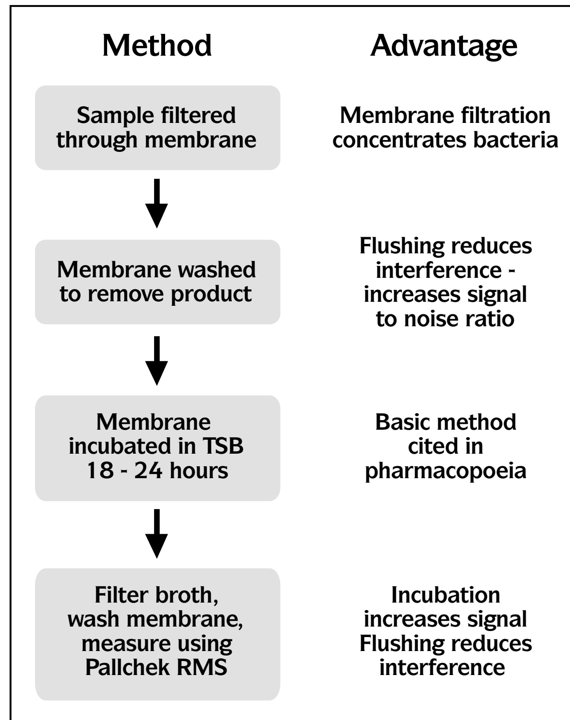
although they are not claimed as sterile, typically they are manufactured using the same aseptic processing strategy of sterile drugs such as injectables and that, in practice, in more than 98% of instances, when tested for microbiological contamination, they are found to be sterile. In those instances where the product is not sterile, an out-of-specification investigation will normally focus on the level of contamination (which is frequently very low, on the order of 10 CFU per container) and the identity of the contaminant.

Therefore, the use of a rapid method capable of detecting the low level of contamination present in this type of product just after manufacturing allows the vast majority of batches manufactured to be released very quickly. For those few batches where contamination is detected, an investigation can commence rapidly. If common components of the apparently contaminated batch were destined for use in other batches of product, these components can be quarantined during the investigation so that the other batches of product are not compromised.

This approach is based on a presence/absence method in which samples are filtered using analysis membranes (see Figure 4), exactly as would be the case for many compendial methods. In outline, the method is as follows:

- Following sample filtration, the analysis membrane can be flushed to minimize any components of the sample that might interfere with ATP bioluminescence.
- The analysis membrane can then be placed into Tryptone Soya Broth (TSB) and incubated in a manner exactly analogous to the compendial method.
- After overnight incubation, a portion of this broth (normally 8 mL from a 10 mL volume) is analysed using the **Pallchek** Rapid Microbiology System following the method for direct measurement of a filtered liquid sample.
- The RLU reading obtained is compared to the background reading obtained for uninoculated samples. For the Pallchek Rapid Microbiology System, this background reading is typically of the order of 10^2 RLU.
- If the RLU reading is equivalent to the background reading, the test has shown absence of microorganisms and the batch of product tested is released. If the reading is above the background, it is likely that the product sample was contaminated and further investigation is required. For the Pallchek Rapid Microbiology System, a contamination level of 1-10 CFU at the start of incubation will result in a reading (after overnight incubation of the sample) of typically 10^6 – 10^7 RLU.

Figure 4. Summary of the Presence/Absence Method for Testing Samples, Based on an Incubation Step in Tryptone Soya Broth (TSB).



In addition to the economic considerations of monitoring manufacturing processes for contamination, there is also an increasing regulatory driver in the form of Process Analytical Technologies (PAT). This is too large a subject to discuss in any detail here. As most readers will be aware, PAT is a system for designing, analysing, and controlling manufacturing processes by making timely (ideally, real time) measurements of critical quality and performance parameters of raw materials (and at key steps within the process) to ensure final product quality.

Current compendial methods for detecting microorganisms fall well short of most 'experts' definition of *timely*. Although microbiologists do not yet have access to true real-time detection methods, this type of strategy reduces detection time to 24 hours or less, meaning that microbiological methods are able to move much closer to the ultimate goals of PAT.

Monitoring Water For Injection

Water For Injection (WFI) is normally sterile and pyrogen-free. Typically, the objective of monitoring WFI is to demonstrate that it meets these two criteria. Again, a presence/absence method can be used, in which samples of WFI are filtered through analysis membranes and tested as described above for finished product. The expectation is that an absence of microorganisms will be demonstrated; if contamination is detected, a follow-up investigation is required.

Environmental Monitoring

Many critical areas in a pharmaceutical manufacturing facility are expected to be essentially sterile (e.g., grade A manufacturing areas at <1 CFU detected). Methods such as swabbing can be used easily with a presence/absence test method. Following swabbing of the surface being monitored, the swab can be transported and the tip of the swab removed and transferred into TSB following the current standard procedures used with the compendial methods. The inoculated TSB sample is then processed as described above. The expected result is absence of (no detectable) microorganisms. If contamination is detected, a follow-up investigation will then ensue.

Other Key Applications

Not all users may want to move directly to using rapid microbiology technology for critical applications such as environmental monitoring or they may want to use a common method across a wide range of applications, both as part of a ROI justification and harmonization of methods, for example. Therefore, system flexibility (i.e., the ability of the system to be used across a broad range of applications) is important.

The following are examples of other routine test procedures typically performed in pharmaceutical manufacturing facilities for which the Pallchek Rapid Microbiology System can be used:

- Qualification of microbial suspensions (both ATCC cultures and wild-type organisms used in a variety of studies, including fertility testing

of growth media, demonstrating the efficacy of antimicrobial agents (as described in USP <51>) analysing the potency of disinfectants and cleaning agents (White et al. 1961) and several others.

For all of these examples, the basic requirement is to confirm that a microbial suspension is within specified limits (i.e., count of microbial cells). As the cultures used are well characterized and controlled, there is a reproducible and consistent relationship between the number of microbial cells in a sample and the amount of ATP in the same sample. It is straightforward to construct correlation curves relating RLU readings to CFU data for appropriate dilutions of these cultures. Labbe (2004) has published this application and the reader is recommended to review this paper for further details of this approach.

- Testing Biological Indicators (BIs) during autoclave validation. Again, a presence/absence approach can be adopted to demonstrate that no recovery has been detected from the BIs used.
- Aseptic media fills.

VALIDATION AND REGULATORY APPROVAL

It is not possible to cover this subject in full detail here. PDA Technical Report #33 (PDA 2000) covers this subject in some detail. In this document, the user is recommended to follow the Equipment Qualification Model (EQM). The EQM is a comprehensive approach that includes Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ). IQ and OQ are normally provided and supported by the system manufacturer. They should also be able to assist the user in several aspects of PQ, including operator training. Ultimately, however, the user has to design and perform their own PQ, since the objective of this is in part to demonstrate the ruggedness and robustness of the system being validated with their own operators in their own facility with their samples.

PDA Technical Report #33 covers several important parameters that should be included in the PQ that forms part of the validation process leading up to the adoption of a rapid microbiology method. Similar attributes are also included in draft USP <1223> (Pharmacopoeial Forum 2005). The key attributes are:

- Accuracy
- Precision
- Specificity

- Limit of detection
- Quantification limit, linearity, and range (for systems that count microorganisms)
- Ruggedness
- Robustness

Definitions of these are given in draft USP <1223> and the reader is referred to this reference for more information.

Draft USP <1223> recommends a comparability approach in which the above attributes of the new rapid method are measured alongside the current compendial method that it is intended to replace. In the case of the Pallchek Rapid Microbiology System, data demonstrating such comparability is provided in the accompanying validation guide for the product and the method used can form the basis of a user's own PQ. Also, in the case of the Pallchek Rapid Microbiology System, data on the ruggedness and robustness of the system over a five-year period of testing the product within Pall's manufacturing facility is included in the validation guide for the product.

The Pallchek Rapid Microbiology System was the first Rapid Microbiology product to be granted approval by FDA CDER for a specific application at GlaxoSmithKline (GSK) for the release of non-sterile pharmaceutical products, thus demonstrating that rapid methods will be accepted by key regulatory authorities if the appropriate justification and supporting data is supplied as part of the submission.

CONCLUSIONS

Although the process for validating and adopting a rapid microbiology method has been made simpler over recent years, it is still a substantial investment for any pharmaceutical manufacturing operation. Protecting this investment is aided by significant supporting information from the sources cited here, by presentations by experts at many rapid microbiology conferences run globally, and increasingly by the regulatory authorities who continue to make substantial efforts to guide and support users wishing to adopt rapid microbiological methods. By using these resources wisely, the perceived risks of adopting new technology can be essentially eliminated and adoption of rapid microbiology simplified and streamlined.

ACKNOWLEDGEMENTS

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ABOUT THE AUTHORS

Lucia Ceresa, Ph.D., obtained her degree from the University of Agriculture in Milan in 1981. She worked on Plant Pathology and her studies of fungicidal activity and structure were published in "*Pesticide Science*" in 1983. She obtained an additional qualification for a one-year course in the analysis and purification of water. She was a teacher for eight years in Chemistry, Biochemistry, Plant Pathology, Microbiology, and Food Industry Technology. During this time, she published three books. She then worked for fours year at Millipore Corporation, in Technical Service and Field Specialist roles, supporting validation study for products utilized for aseptic productions.

Lucia Ceresa joined Pall Corporation in 1996. Since then, she has gained valuable experience in filtration technology for the production and quality control of pharmaceutical products. She is now the European Marketing Manager for Rapid Microbiology at Pall Life Sciences, based in Italy.

Dr. Peter Ball obtained a BSc in Microbiology from the University of London in 1975 and a PhD for research in Molecular Biology from the University of Bristol in 1979. From 1978, he lectured and conducted research at the University of Bristol, until he joined Pall Corporation in 1981. During his time at Pall, he has worked in the Scientific and Laboratory Services and Research and Development groups. From 2001 until late 2004, he was Director of Strategic Marketing, guiding Pall in its efforts to enter new markets in Biotechnology. He is now Director of Business Development, New Technologies, Pall Life Sciences, based in the U.K.