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The Development of Compendial Rapid Sterility Tests

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ABSTRACT An Expert Panel was formed under the USP General Chapters—Microbiology Expert Committee to provide recommendations on user requirements specifications (URS) and candidate technologies based on the URS in the area of rapid sterility tests. The Expert Panel provided recommendations for the critical URS for candidate rapid sterility tests, which were: 1) the ability to detect a wide range of microorganisms, i.e., specificity; 2) detection of a low number of microorganisms, i.e., limit of detection; 3) time to result; 4) improved patient safety; 5) sample preparation; and 6) sample quantity, i.e., minimum number of articles tested and quantity per container tested. Based on a review of these user requirements, the Expert Panel recommended that adenosine triphosphate bioluminescence, flow cytometry, isothermal microcalorimetry, nucleic acid amplification, respiration, and solid-phase cytometry advance as candidates for proof-of-concept studies to develop risk-based compendial rapid sterility tests.

INTRODUCTION

It is widely recognized that the current growth-based sterility tests in *Sterility Tests* ⟨71⟩ (*1*) with at least a 14-day incubation period are not suitable for short-lived products or those prepared for immediate use or administered to patients before the completion of the compendial sterility test. To address the needs of stakeholders making compounded sterile preparations, radiopharmaceuticals, and cell and gene therapies, the USP Microbiology Expert Committee has begun work on the development of a new generation of rapid compendial sterility tests.

BACKGROUND

With the primary consideration of improved patient safety, the Expert Panel began by establishing the user requirements specifications (URS) for rapid sterility tests. The consensus reached was that not all URS were the same for four main stakeholder groups indicated above. Therefore, URS were established for: 1) sterile compounding; 2) positron emission tomography (PET) drugs and other short-lived radiopharmaceuticals; 3) cell therapy; and 4) traditional pharmaceutical manufacturing.

Once the URS were established, the Expert Panel recommended the most suitable technologies or analytical platforms as candidates for a compendial rapid sterility test for proof-of-concept studies. Where analytical platforms were dependent upon instruments and reagents supplied by vendors, only non-proprietary technologies marketed by two or more instrument manufacturers were considered as potential candidates. The Expert Panel acknowledges that one or more of these analytical platforms may be found to have insurmountable technical limitations, which may prevent them from becoming compendial test methods. Despite being compendial tests, the rapid sterility tests would need to meet method suitability testing requirements for each

pharmaceutical and biological product and would be subject to review in their regulatory submissions.

HISTORY OF USP STERILITY TESTS

It is useful to review the history of how the USP sterility tests evolved (*2–4*). *Table 1* contains a brief summary of the development of ⟨71⟩ from 1936–2009.

Table 1. The Evolution of USP Sterility Tests

Compendial Revision	Brief Description of the Sterility Test
USP XI (1936), page 469, *Tests for the Sterility of Liquids*	7-day incubation at 37° in a beef extract-peptone-dextrose broth
USP XII (1941), *Sterility Test for Solids* added	Additions: broth for sterility tests under anaerobiosis, inactivating fluids, and a honey medium for molds and yeast incubated at 22° –25° for 15 days
USP XVII (1965), pages 829–832, *Sterility Test*	Additions: fluid thioglycollate medium incubated at 30°–32° for 7 days, fluid Sabouraud dextrose medium incubated at 22°–25° for 10 days, and bacteriostasis and fungistasis testing added to demonstrate the suitability of the method for each specific product
USP XVIII (1970), pages 851–857, *Sterility Tests* ⟨71⟩	Revisions: fluid thioglycollate medium incubated at 30–35° for 14 days for aseptically filled products and 7 days for terminally sterilized products; soybean-casein digest medium incubated at 20° –25° for aseptically filled products and 7 days for terminally sterilized products; and the incubation period reduced from 14 to 7 days for membrane filtration sterility tests
USP 27 (2004) pages 2157–2162, *Sterility Tests* ⟨71⟩	Harmonization: effective January 1, 2004; however, the compendial sterility tests contained 11 local non-harmonized requirements; all incubation times, regardless of product, were 14 days
First Supplement to *USP 32* (2009), *Sterility Tests* ⟨71⟩	Revisions: the 11 local non-harmonized requirements were removed with an official date of August 1, 2009

LIMITATIONS OF THE SELECTED MEDIA

In general, microorganisms that are found in pharmaceutical drug products are present in low numbers and under stressed conditions due to 1) product formulation (especially the presence of antimicrobial agents or active ingredients); 2) manufacturing processes; and 3) physicochemical conditions such as low nutrient levels, pH (deviating from neutral), low-water activities, and exposure to temperatures above or below ambient temperature. To proliferate in microbiological growth media, microorganisms need to repair stress-induced damage, activate different biosynthetic and metabolic pathways, and acclimate to the media before they can enter a logarithmic growth phase.

Despite the belief that the sterility test media can support the growth of low numbers of stressed microbial cells, the media selection and incubation conditions of the compendial sterility test may not be optimal and may, in fact, be seriously compromised in an attempt to isolate the widest range of microorganisms (*2*). For example, fluid thioglycollate medium may be considered suboptimal for 1) strict and facultative anaerobes due to its aerobic incubation, 2) bacterial and fungal spore germination and growth, and 3) vegetative bacteria and fungi due to its low redox

potential, medium viscosity, and component toxicity. Soybean–casein digest medium may be compromised for the isolation of skin-derived bacteria by the low incubation temperature, i.e., 20°–25°.

The unintended selectivity of the sterility test is illustrated by the common finding that the majority of sterility failures occur in only one of the two media when the microorganisms are capable of growth in both media. For example, 55% of the sterility failures had growth in the soybean-casein digest medium only, 39% grew in the fluid thioglycollate medium, and a mere 9% grew in both media (*5*) with over 30% of growth occurring between 7 and 14 days of incubation (*6*).

SAMPLE SIZE LIMITATIONS

Chapter ⟨71⟩ defines the quantities of a pharmaceutical drug product per container to be tested per media and the number of units based on the batch size (see *Sterility Tests* ⟨71⟩*, Table 2* and *3*, respectively). The number of vials tested is a usually 20 or 40 units, depending on the fill volume of the containers. Considering the statistical power of the sample size with respect to a typical batch size in excess of 30,000 vials, the test is not capable of detecting a low microbial contamination rate associated with aseptically filled sterile drug products, i.e., there is only an 18% chance of detecting a 1% contamination rate (*4*) (see *Table 2* below).

Table 2. Probability of Failing the USP Sterility Test with Required Sample Size

Frequency of Contaminated Units in a Batch	**Probability of Failing the USP Sterility Test with Required Sample Size (***Sterility Tests* ⟨71⟩*, Table 2* **and** *3***)**
1 in 1000	0.0198 (2%)
5 in 1000	0.0952 (9.5%)
1 in 100	0.1813 (18%)
5 in 100	0.6321 (63.2%)
1 in 10	0.8647 (86.5%)
5 in 10	1.000 (100%)

Furthermore, there are additional challenges with compounded sterile preparations, short-lived radiopharmaceuticals, and cell therapies compared to most pharmaceutical drug products. The lot sizes are usually small, the products must be used promptly, and sampling will deplete a significant portion of each lot, causing an economic loss and reduced availability of the material for patient treatment.

Currently, the minimum number of articles tested and quantity per container tested per media are defined in *Sterility Tests* ⟨71⟩*, Table 2* and *3*. This sampling plan is suitable for manufactured pharmaceuticals, but it depletes the batch, and is therefore unsuitable for products generated by sterile compounding pharmacies, PET facilities, and cell therapy centers because of their small batch size and the therapeutic value of the product to the individual patient. A further consideration is the sample size limitation of these advanced technologies.

Alternative sampling plans have been proposed in compendial chapters. The recommended approaches to sterility testing of cell therapy products can be found in *European Pharmacopoeia* (*EP*) 2.6.27 for batch sizes less than 40 units.

The *EP* provides 2.6.27 *Microbiological Examination of Cell-based Preparations* to use for cell therapies when the tests in 2.6.1 *Sterility* cannot be performed. These limitations may be due to the nature of the preparation, the process steps during which microbial contamination may be introduced, the short shelf-life of cell therapy products, the amounts available for testing, and sampling-related issues. *EP* positioned this test, not strictly as a sterility test, but as a test to screen for microbial contamination that may be better suited for certain situations. The chapter points out that with the use of a single donor or manufacturing-related capacity restraints, the sample volume available for testing may be limited. Microbial contamination can be missed if the sample size is not sufficient to ensure suitable sensitivity and specificity of the chosen test method.

The sample size for cell-based preparations, where the total infusible volume (V) is between 1 mL and 1 L in a single unit, is given in *Table 3* below.

Table 3. *European Pharmacopoeia* 2.6.27 Recommended Sample Sizes

Cell-Based Preparation Volume (mL)	Total Test Sample Volume
$10 \le V < 1000$[a]	1% of the total volume
$1 \le V < 10$	100 µL
$V < 1$	NA

[a] V is total infusible volume.

In a manner similar to cell-therapy preparations, the sample quantity and sampling plan for PET radiopharmaceuticals must also accommodate the limited number of vials (usually 1) and the volume of product produced in a batch (usually less than 15 mL). If the batch consists of a single container, the sterility test sample size must be at least 1% of the total batch volume. For example, if a batch consists of 1 vial containing 15 mL, use at least 0.15 mL for purposes of the sterility test. If the batch consists of more than one container, use a volume from a single container that represents at least 1% of the total batch volume. If a batch consists of 3 vials each containing 25 mL, use at least 0.75 mL from 1 vial for purposes of the sterility test.

USER REQUIREMENT SPECIFICATIONS

Based on the work of the USP Expert Panel, 15 major user requirement specifications of different stakeholders were considered:

- Ability to detect a wide range of microorganisms, i.e., specificity
- Availability of instruments and reagents from multiple vendors
- Availability of Reference Standards
- Data integrity
- Ease of use/simplicity of test and data interpretation
- Low false-positive and false-negative rates
- Limit of detection
- Method suitability
- Improved patient safety
- Regulatory acceptance
- Robustness and reliability of equipment
- Sample preparation

- Sample quantity, i.e., minimum number of articles tested and quantity per container tested
- Time to result
- Aseptic test material handling, i.e., open vs. closed systems

DETAILED DESCRIPTIONS OF THE MOST CHALLENGING USER REQUIREMENTS

Challenging user requirements specific to one or more stakeholder groups are:

- Ability to detect a wide range of microorganisms, i.e., specificity
- Limit of detection
- Time to result
- Improved patient safety
- Sample quantity i.e., minimum number of articles tested and quantity per container tested
- Sample preparation
- Aseptic test material handling, i.e., open vs. closed systems

The user requirements listed above will be discussed in more detail below.

Ability to Detect a Wide Range of Microorganisms

Although all the analytical platforms should have the ability to detect a wide range of bacteria, yeast, and mold, it is equally important to demonstrate that the rapid sterility test technology chosen is capable of detecting microorganisms implicated in sterility test failures, infection outbreaks, and product recalls associated with either compounded sterile preparations, radiopharmaceuticals, cell therapies, or manufactured pharmaceuticals. This is especially true if the technology, after risk analysis, is shown to improve patient safety with the administration of the products unique to that stakeholder group.

For example, a 2014 report from The Pew Charitable Trusts documented over 25 pharmacy compounding errors, the majority being microbial contamination associated, with 1,049 adverse events and 89 deaths since 2001. The report identified the bacterium *Serratia marcescens* as most frequently implicated in compounded sterile preparation infections (*7*). In addition, a prospective, nationwide surveillance study of nosocomial bloodstream infections from U.S. hospitals over a 7-year period (*8*) implicated coagulase-negative staphylococci (31%), *Staphylococcus aureus* (20%), *Enterococcus* species (9%), *Candida* species (5%), *Escherichia coli* (3%), *Klebsiella* species (2%), and *Pseudomonas aeruginosa* (2%). The absence of strict anaerobes among microorganisms most responsible for bloodstream infections is notable and is considered to be due to the high levels of oxygenation of blood.

Limit of Detection

Within the limitations of preparing inocula with one or more colony-forming units (cfu), growth-based sterility tests can be shown to have at least a theoretical limit of detection (LOD) of 1 cfu or 3 cfu based on a Poisson distribution. Setting an LOD of a single viable cell with all technologies is an unrealistic barrier to entry for any sterility test, especially when the signal is not the colony-forming unit that is amplified by cultural enrichment. The concept of an infectious dose is well established, especially in food and clinical microbiology (*9*). Although the absence of viable microorganisms in the product has generally been accepted as a definition of sterility,

there is little or no evidence that 1 cfu is an infectious dose (i.e., clinically significant) for injectable products. To the contrary, well-established evidence from the study of infection rates due to the administration of platelet concentrates to human cancer patients suggests that the infectious dose may be 10^2–10^3 viable microorganisms, depending on the virulence of the microorganism. The study of transfusion infection with platelet concentrations provides an excellent test case to determine the infectious dose as they have an estimated contamination rate between 0.03% and 0.7%. In a unique study, Jacob et al (2008) determined the bacterial content of thousands of platelet concentrates immediately prior to infusion (*10*) and found that a detection threshold of at least 10^3 cfu/mL would detect more than 95% of all infection cases and that a detection threshold of 10^2 cfu/mL would detect all cases (100%). These general findings were confirmed in a follow-up publication from the same researchers from Case Western University (*11*) and are generally accepted by the transfusion microbiology community (*12*).

As noted by the authors of a recent study on the use of the 16S rRNA polymerase chain reaction (PCR) sterility test for stem cells with the demonstrated bacterial sensitivity of 10–100 cfu/mL, a test method with a sensitivity of 100 cfu/mL would be suitable to detect clinically significant bacterial contamination of blood and cell products (*13*).

Time to Result

The incubation time for growth-based ⟨71⟩ sterility tests is at least 14 days; this makes it unsuitable for PET and cell therapy as these short-life products would be administered before completion of the test. This time to result is marginally acceptable for sterile compounding, but generally suitable for pharmaceutical manufacturing. Some PET drugs may be administered immediately after preparation due to the short half-life of certain PET radionuclides, so a sterility test needs to be real time for this stakeholder group. The most commonly used PET radionuclide is fluorine-18, which is normally used within 12 h. For compounded sterile preparations and cell therapies, sterility tests need to be completed within a maximum of 48 h, especially when the dose is needed promptly for a waiting patient. Additionally, manufactured pharmaceuticals can be tested within 5–7 days to shorten the batch release cycle time. See *Table 4* for typical expiration dating.

Table 4. Typical Beyond Use/Expiration Dating of the Stakeholder Products

Stakeholders	Representative Products	Beyond Use/Expiration Dating
Sterile compounding pharmacies	Low Risk: Reconstitution and transfer of a 1-g vial of cefazolin into an IV bag Medium Risk: Distribution from a 10-g bulk pharmacy vial of vancomycin among several final doses High Risk: Patient-controlled analgesic from powdered morphine	Low Risk: 48 h (room temperature); 14 days (2°–8°); 45 days (frozen) Medium Risk: 30 h (room temperature); 9 days (2°–8°); 45 days (frozen) High Risk: 24 h (room temperature); 3 days (2°–8°); 45 days (frozen)
PET facilities	Fluorine-18 fluorodeoxyglucose (half-life of 110 min)	Cellular therapy products may be transported for administration in hours or days without cryopreservation, or stored in a cryopreserved state (<−30°) indefinitely.
Cell therapy facilities	Stem cells	Cellular therapy products may be transported for administration in hours or days without cryopreservation, or stored in a cryopreserved state (<−30°) indefinitely.
Pharmaceutical manufacturers	Numerous examples	2–3 years at ambient or refrigeration temperature

[NOTE—Signals employed by different technologies may be amplified by enrichment culture with 24–48 h incubation or by concentration, e.g., filtration, selective adsorption and elution, or centrifugation, to reduce the time to result and lower the limit of detection.]

Improved Patient Safety

It is widely accepted that a rapid sterility test for compounded sterile preparations, radiopharmaceuticals, and cell therapies will improve patient safety, especially if contaminated materials can be detected before administration to patients. Furthermore, sterility test methods that continuously monitor for the presence of viable microorganisms during processing as a control strategy would be advantageous. Such monitoring after product release, with a reporting mechanism when a failure is detected, would enable the laboratory to alert the clinician, who could then intervene as necessary to protect the patient. The ability of a bacterial contaminant to grow in a product and its virulence when infused into a patient should both be considered.

Other limitations of the compendial sterility test methods that may impact patient safety are as follows:

- The ability of the sterility test to be affected by antibiotics in the test sample
- The subjectivity of detecting microbial growth in microbiological culture broth
- The lack of detection of culture-negative infectious agents
- The unintended selectivity of culture media and the incubation temperature/conditions

Many compounded sterile preparations are antibiotics. Cell cultures used to produce cell and gene therapies may include antibiotics to control microbial contamination during aseptic

manipulations such as cell culture expansion. As the mode of action of antibiotics usually involves the bacterial cell wall or protein synthesis, residual antibiotics in the sterility test media may inhibit bacterial growth leading to false-negative test results. Sterility test methods that are not growth-based generally are not affected by antibiotic residuals.

Microbial growth in broth will appear as turbidity, pellicles, floccular growth, or precipitation. However, the product may obscure the presence of microbial growth. It is estimated that cell densities exceeding 10^6 cfu/mL are needed to make the media turbid for detection by the naked eye. These assessments are highly subjective and that may result in false-negative test results.

Although rare, culture-negative infections are observed in clinical microbiology, and PCR and 16S rRNA gene sequencing have been used for bacterial detection and identification, e.g., Whipple's disease (*14*).

The sterility test media may be incapable of detecting a contaminated product. For example, in 2002 and 2003 there were three clusters of three outbreaks of clostridial disease caused by *Clostridium sordelli* in cows and sheep in Spain. Ironically, the outbreaks were linked to anti-clostridial vaccines, all produced by the same manufacturer, that were intrinsically contaminated with the same strain of *C. sordelli* (*15*). The vaccine batches were released to the market using the harmonized sterility test. The majority of vials (93%) from the implicated batches contained low counts of *C. sordelli* when cultured on sulfite-polymyxin-sulfadiazine agar incubated under anaerobic conditions at 37° for up to 60 days. The fluid thioglycollate medium used in the sterility test failed to detect the clostridial contamination, presumably due to thioglycollate inhibition and the shorter incubation time.

Sample Quantity

The minimum number of articles tested and quantity per container tested per media are defined in *Sterility Tests* ⟨71⟩*, Table 2* and *3*. Whereas this sampling plan is suitable for manufactured pharmaceuticals, it is unsuitable for products generated by sterile compounding pharmacies, PET facilities, and cell therapy centers because of their small batch size, high cost, and therapeutic value to the individual patient. A further consideration is the sample size limitation of the advanced technology (see *Table 6*). Alternative sampling plans have been proposed, as discussed in *Sample Size Limitations* above.

Sample Preparation

The complexity and number of steps in the sample preparation process add to the analyst's hands-on time, as well as the overall reduced recovery of the signal of viable microbial cells. Furthermore, to obtain a high throughput and short time to results, one needs an easy sample preparation. However, a complex sample preparation may be acceptable if the method provides improvements in time to results and LOD or has the potential to be automated. PET drugs and radiopharmaceuticals have added requirements associated with the safe handling of radioactive materials and the need for effective shielding to reduce radiation exposure to acceptable levels.

Aseptic Test Material Handling: Open vs. Closed Systems

Advanced technologies with closed systems will mitigate risk of microbial contamination with live organisms or their artifacts, such as adenosine triphosphate (ATP) or nucleic acid. With open

systems, a decision may be made at the testing laboratory to conduct the testing in an isolator system, which adds to the expense and reduces testing throughput.

POTENTIAL TRADE-OFF BETWEEN CONFLICTING USER REQUIREMENTS

There are obvious trade-offs between LOD, sample size, and time to results (see *Table 6*). Detecting a contaminated unit prior to administration is paramount in improving patient safety, therefore the proposed compendial rapid sterility tests must be risk-based with the stakeholder selecting the technology that best serves the interests of their patients and the beyond-use dating of their products. For example, patient safety may be served even if the LOD is 10–100 viable microbial cells, if the test can be completed the same day that a low-volume radiotracer is compounded.

EXPERIENCE WITH BACTERIALLY CONTAMINATED PLATELET CONCENTRATES

The collective experience with the administration of human platelet concentrates is revealing. This cellular component, which is obtained from whole blood collection or apheresis, is stored on rocking platforms at ambient temperature for up to 7 days prior to transfusion. These units have been reported to have bacterial contamination rates of 0.05%–0.2%. Based on the measurement of the contamination of transfused platelet concentration it was apparent the rates of septic reactions were about 50 times less and fatality rates were about 250 times lower (see *Table 5*). This supports the view that an infectious intravenous dose is not 1 cfu but the order of 10–100 CFU as reported by Jacobs, et al. (*10*).

Table 5. Rates of Contamination, Septic Reactions, and Deaths with Administration of Platelet Concentrates

Contamination Rate per Units Transfused	Rate of Septic Reactions per Unit Transfused	Fatality Rate per Units Transfused
0.05% (5000 contaminated units/million units transfused annually)	1%–1.3% of the contaminated units (10–13 septic reactions/million units)	15%–20% of the septic reactions (2 deaths/million units)

(From the FDA Draft Guidance for Industry: "Bacterial Risk Control Strategies for Blood Collection Establishments and Transfusion Services to Enhance the Safety and Availability of Platelets for Transfusion", March 2016)

SELECTION OF AVAILABLE TECHNOLOGIES WITH POTENTIAL FOR USE AS A RAPID STERILITY TEST

The Expert Panel selected the following six analytical platforms, listed alphabetically, as candidates for compendial rapid sterility testing:

- Adenosine triphosphate bioluminescence
- Flow cytometry
- Isothermal microcalorimetry
- Nucleic acid amplification
- Respiration
- Solid phase cytometry

Brief Descriptions of the Six Analytical Platforms

Each of these candidate advanced analytical platforms is briefly discussed below, and key references are provided. For an overview, the reader is referred to the 4-volume series of the *Encyclopedia of Rapid Microbiological Methods* (*16*) and a book dedicated to the topic, *Rapid Sterility Testing* (*17*).

Adenosine Triphosphate Bioluminescence

This is a well-established technology that uses luminometers and reagents available from multiple instrument manufacturers. The energy from living cells is stored as ATP, which can be measured as light when exposed to luciferase from the American firefly. Each ATP molecule consumed by luciferase produces 1 photon of light. The result detected by a luminometer is typically expressed in relative light units (RLU) and is instrument-, reagent-, and organism-dependent. The ATP content of different microorganisms ranges from $2–4 \times 10^{-18}$ mol/cfu for Gram-negative bacteria, $5–8 \times 10^{-18}$ mol/cfu for Gram-positive bacteria, and $300–800 \times 10^{-18}$ mol/cfu for fungi (*18*). Given the high signal-to-noise ratio, the microbiologically relevant instrument detection limit is on the order of 5000 RLU, equivalent to 10^3 cfu.

This LOD will detect the presence of microorganisms at 3–4 log lower numbers within an aliquot of the media than that required for visual detection of growth in the media. For a sterility test, an enrichment culture, either in liquid media or on a membrane filter on solid media, could be used with an incubation time of 2–7 days.

Flow Cytometry

Flow cytometry may be used to detect fluorescently labeled viable microbial cells after an enrichment culture step that takes 24–48 h (*19*). A labeling reagent consisting of either a fluorogenic substrate or vital stain is used to differentiate viable cells from dead cells and cellular debris. Cell viability is indicated by the ability of the intact cell membrane to retain a fluorochrome generated by non-specific cellular esterase, or by labeling the cell with nucleic acid-specific vital stain. An argon laser illuminates each cell in the flow stream and the emitted light is detected by a dual photomultiplier array. The signal is digitized and interpreted by discrimination software. Instrumentation and reagents may be obtained from multiple vendors. The LOD for this technology may be, in the best case scenario, 10–100 viable microbial cells in the absence of a high-particulate background, so an enrichment/concentration step would be necessary unless a higher LOD than 1 cfu is accepted.

Isothermal Microcalorimetry

Isothermal microcalorimeters monitor enthalpy changes in closed vials (systems) related to microbial metabolic activity and growth. With current instruments, 10^4 active microbial cells can release enough heat to be detected, although enrichment is needed for detection (2–7 days to result). The system has its origin in the cement and explosive industry. Within the past several years its use in biology started to receive more attention, and it is being applied in geology (e.g., soil testing), parasitology, optimization of fermenting processes, the food industry (e.g., monitoring of microbial growth in milk fermentation processes), clinical applications, and dentistry (*20*). Recently, the application of isothermal microcalorimetry in pharmaceutical microbiology has also been evaluated (*21*).

Nucleic Acid Amplification

Real-time quantitative PCR has the potential to monitor the exponential phase of PCR through 36–48 cycles of amplification using universal primers to estimate the initial quantity of the target DNA, which is in turn proportional to the number of microbial cells in the test sample. Unlike DNA, cellular RNA has a rapid metabolic turnover and is a better indicator of viable microorganisms. For example, *E. coli* contains 2 molecules of DNA and 20,000 molecules of 16S rRNA/cell (*22*). This process is achieved by the conversion of RNA into a complimentary copy of DNA by the enzyme reverse transcriptase and can be analyzed in real time in either a quantitative assay (enumeration test) or qualitative assay (sterility test). Alternatively, for DNA-based PCR, a sample pre-treatment with ethidium monoazide or propidium monoazide may allow for differentiation between live and dead microbial cells (*23*, *24*).

Realistically, an LOD of 1 viable cell is probably an insurmountable challenge, especially for a test that relies on a DNA/RNA target and universal primers.

Generally, the LOD ranges from 10–1000 viable cells/mL of sample, and in some reported cases it ranges from 10–100 viable cells/mL. Recently it was shown that PCR may actually achieve detection of microorganisms with a limit of 10^2–10^3 cfu/mL in a sample containing a high concentration of up to 10^6 mammalian cells/mL without the need for pre-incubation in microbial growth media (*25*). Adding a growth-based enrichment step for at least 24–48 h and comparing the PCR results before and after enrichment may provide a practical solution for sterility testing. Alternatively, concentration methods could be applied to enrich the sample and reduce the sample volume. Instrumentation and reagents may be sourced from multiple vendors.

The higher LOD of 10–100 viable cells/mL does not mean that PCR methods are unsuitable for sterility testing. Jacobs, et al. (*10*) reported the relationship between the bacterial load and transfusion reactions with platelet concentrates. Based on the data reported they conclude that a threshold of 1000 cfu/mL would detect more than 95% of all cases of contamination and 90% of the reactions, whereas a 100 cfu/mL threshold would detect all cases (100%). Data derived from transfusion medicine are particularly useful (see *Table 5*), and are used for patients undergoing bone marrow transplantation or receiving chemotherapy.

Use of non-growth based sterility tests such as PCR increases patient safety for the following reasons:

- With sterility testing that is close to real-time, the test is completed before the short-lived product is infused into a patient
- Culture-negative infectious agents are isolated
- The test is unaffected by antibiotics in the test sample
- The test is less sensitive to background noise resulting from animal cell lysis (e.g., particles, ATP), as compared to other technologies, because specific microbial genes are targeted

Respiration

This broad category ranges from classical respirometers to gaseous headspace analyzers to automated blood culture systems. The use of automated blood culture systems has been successfully extended to sterility testing of cell therapy products. In 2004, the FDA approved a supplement to the biologics license held by Genzyme Biosurgery for Carticel, autologous cultured

chondrocytes, to use the BacT/ALERT™ Microbial Detection System with a 7-day incubation as an alternative to the compendial sterility test for lot release (*26*).

Other instruments are available to detect and enumerate respiring microorganisms. For example, tunable diode laser absorption spectroscopy (TDLAS) can measure O_2 depletion or CO_2 increase in closed units containing growing microorganisms in culture medium. The system was developed to monitor gas headspace composition in closed units and also could be used for automatic media fill inspection (*21*, *27*). TDLAS has gaseous calibration standards, and minor adaptations are needed if the system is to be used for sterility testing (e.g., calibrating for higher-volume containers).

Note that all the systems of the respiration platform require microbial growth and metabolic activity for detection, i.e., the usual time to result of 2–7 days is required. However, the results can be progressively monitored to detect a sterility test failure earlier in the incubation period, which is a huge advantage with short-life products.

Solid-Phase Cytometry

Several instrument manufacturers market systems based on solid-phase cytometry. For instance, the ScanRDI™ microbial analysis system has the most market experience and combines fluorescent labeling and solid-phase laser scanning cytometry to rapidly enumerate viable microorganisms in filterable liquids (*28*). Cells are collected by filtration on 0.45-µm polyester membranes and treated with background and viability stains. The filters are scanned in a cytometer by a high-speed, 488-nm argon laser. Fluorescence is detected by multiple photomultiplier tubes and processed to differentiate between labeled microorganisms and background noise. The scan is displayed as map that identifies the positions of the fluorescent events, which are verified using an epifluorescence microscope with an automated motorized stage to locate the individual events. The system is claimed to identify individual viable microorganisms in 2–3 h.

In *Table 6*, the critical operating parameters of representative candidate modern microbiological methods are provided for informational purposes. These values are estimated, and may be optimistic in some cases. The list is not all-inclusive and does not constitute an endorsement of any single technology.

Table 6. Operational Parameters of Candidate Technologies

Representative Detection System	Technology	Limit of Detection (cfu/mL)	Time to Result	Sample Size (mL)
Gram stain	Classical	10^4–10^5	30 min	0.1
BacT/ALERT System	Respiration	1–10	Overnight to 7 days	5–10
ScanRDI System	Solid-phase cytometry	1–10	2–3 h	1–500
Milliflex Rapid System	ATP bioluminescence	1–10	5–7 days	1–500
FACS analysis	Flow cytometry	10–100	6–8 h (pre-enrichment)	0.1–2
Roche LightCycler	Nucleic acid amplification	1–100	2–4 h	0.2–2
TAM V	Isothermal microcalorimetry	1–10	2–7 days	1

Representative Instrumentation Manufacturers of the Candidate Technologies

One requirement for an analytical platform to be considered as a compendial sterility test is that it is nonproprietary and there are multiple vendors for the technology and associated reagents. Although it is not all-inclusive, *Table 7* provides more details of the justification based on this requirement.

Table 7. Commercially Available Instrumentation Showing Multiple Vendors

Advanced Technology	Instrument Name	Vendor
ATP bioluminescence	Biotrace 2000 Pallchek Rapid System Milliflex Rapid System Celsis RapiScan BioMAYTECTOR	Biotrace International, Bridgend, UK Pall Corp., Port Washington, NY Millipore Corp., Bedford, MA Charles River Laboratories, Inc., Wilmington, MA Hitachi Plant Technologies, Tokyo, Japan
Flow cytometry	Bact-Flow FACSMicroCount	bioMerieux, Hazelwood, MO Becton, Dickinson & Co. (BD), Sparks, DE
Isothermal microcalorimetry	TAM III calorimeter Biocal 2000 isothermal calorimeter 48-channel isothermal microcalorimeter	TA Instruments, Wilmington, DE Calmetrix, Arlington, MA SymCell Sverige, Kista, Sweden
Nucleic acid amplification	Multiple thermocyclers and amplicor analyzers	Roche Applied Science, Indianapolis, IN Applied Biosystems, Foster City, CA Cepheid, Sunnyvale, CA
Respiration	Promex 4200 microrespirator BACTEC System BioLumix BacT/ALERT 3D Dual-T System Pall eBDS System TDLS	PromChem Ltd., Edenbridge, UK BD Diagnostics, Sparks, DE BioLumix, Ann Arbor, MI bioMerieux, Hazelwood, MO Pall Corp., Port Washington, NY Lighthouse Instruments, Charlottesville, VA
Solid-phase cytometry	ScanRDI System BioSafe PTS MuScan System	bioMerieux, Hazelwood, MO Charles River Laboratories, Inc., Wilmington, MA Innosieve Diagnostics, Wageningen, The Netherlands

The path forward for the adoption of these analytical platforms as compendial tests for short-lived products includes 1) writing an informational general chapter on risk-based sterility testing, 2) collaborative development of generic rapid sterility tests and validation of the selected test methods, and 3) writing and publishing them as official *USP* tests.

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[d] The conflicts of interest of the named authors of this article are as follows: Yongqiang Zhang is employed by BD Biosciences; Felix Montero Julian is employed by bioMerieux ; Anthony M. Cundell and Michael J. Miller consult with some of the technology vendors indicated in this article

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