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▲⟨1071⟩ RAPID MICROBIAL TESTS FOR RELEASE OF STERILE SHORT-LIFE PRODUCTS: A RISK-BASED APPROACH

INTRODUCTION

It is widely recognized that the current growth-based sterility tests with an incubation period of at least 14 days (see *Sterility Tests* ⟨71⟩) are not suitable for products with a short shelf-life or for products prepared for immediate use, which are usually infused into patients before the completion of the test (*1*). These short-life products include compounded sterile preparations (CSPs), positron emission tomographic (PET) products, and cell and gene therapies, which require a new generation of risk-based approaches that include rapid microbial tests. For a general discussion of the factors other than sterility testing that contribute to sterility assurance, see *Sterility Assurance* ⟨1211⟩. It should be noted that as with alternate test methods, the referee test in the event of a dispute is ⟨71⟩.

If a microbial test is conducted, patient safety is best served through the completion of a test that detects microbial contamination prior to product use.

The rapid microbial tests (RMTs) should be risk-based so the stakeholder can select the preferred technology for their intended use and balance user requirement specifications (URS) including time to result, specificity, limit of detection (LOD), sample size, and product attributes. For example, many radiopharmaceuticals, due to the short half-life of radiotracers, would benefit most from a real-time microbial test, while CSPs and cell therapy products, due to their short beyond-use dating, would benefit from an overnight test or at least one that is completed within 48 h.

This general informational chapter discusses the needs of those who manufacture/prepare and test products with a short shelf life and their URS, and includes a brief discussion of some suitable methods for risk-based rapid microbial testing for the release of short shelf-life sterile products (hereafter referred to in this chapter as "short-life products").

USER REQUIREMENT SPECIFICATIONS FOR A RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

Selection of an appropriate technology for the rapid microbial testing for the release of a short-life product should be a risk-based decision. The URS (*2*) of different technologies include:

- As short as possible time to result, ideally in real time or less than 24 hours, preferably prior to the administration of the product
- Ability to detect, preferably less than 100 colony-forming units (cfu) in the test sample
- Ability to detect a wide range of viable microorganisms in a product
- Sample quantity, i.e., minimum number of articles tested and quantity per container tested that does not consume a large proportion of the available product; whenever feasible, manufacturers should consider assay requirements during process design
- Aseptic test material handling, i.e., closed systems to reduce inadvertent contamination during testing
- Availability of instruments and reagents from multiple vendors
- Availability of reference standards, negative and positive controls, appropriate for technologies that use signals other than the colony-forming unit
- Ease of use/simplicity of test and data interpretation
- Low rates of false positive and false negative results
- A method suitability testing strategy for each specific product
- Improved patient safety arising from:

- Completion of the test prior to administration
- Tests that provide progressive monitoring and reports of a detection of sterility test failure

- Ability to identify the detected microorganisms, which may be useful to the clinician administering the products and investigating to determine its source
- Robustness and reliability of equipment and reagents used in the testing
- Sample preparation suitable for both manual and automated methods

THE CONCEPT OF RISK-BASED MICROBIOLOGICAL MONITORING AND RELEASE TESTING

A review of the URS for an RMT shows that some risk-based decisions would need to be made especially in terms of time to results, LOD, sample size, and range of microbes detected to allow the use of such tests prior to administration. The stakeholders should carry out a risk assessment for choosing an RMT in cases where the current compendial sterility test is unsuitable.

Benefits may include, for instance:

- Use of RMT in cases where a substantial risk to patient survival exists when the product is administered too late. One striking example from the clinical literature is that of bloodstream infections that are rapidly progressive infections with mortality rates of up to 40% and in which each day of delay in administrating antibiotics leads to a 10% increase in mortality (*3*). In these cases, patient safety is clearly promoted by the completion of a microbial test prior to the administration.
- Use of growth-based RMTs with continuous reading applied for "negative to date" on risk release because fast growing microorganisms can be detected earlier, and if a failure is detected this would enable the clinician to intervene sooner with the patient. When a contaminated product is detected, the laboratory supervisor could inform the clinician treating the patient and intravascular fluid resuscitation and antibiotic treatment may be initiated to avoid septic shock. A sterility test that progressively monitors and automatically reports any failure will therefore have additional advantages as compared to a single reading at the end of the incubation period.
- Use of non-growth based RMTs with LOD above 1 cfu that is amplified by growth in microbiological media but very fast time to results. The ability to detect contamination, in real time, prior to the administration of the short-life product may be considered more important than detection of a single colony-forming unit in the product. When considering risk to the patient, choice of the RMT should factor in the sensitivity of the assay versus the time to detection. Assays should be reasonably sensitive to detect the presence of a low-level contaminant and should do so in a time frame that allows results to be available before product administration.
- Other advantages of nongrowth-based RMT methods also may include the inability to be affected by antibiotics in the test sample and the detection of culture-negative infectious agents. Some DNA–targeted antibiotics (e.g., polymyxin B and bacitracin) have been shown to inhibit PCR amplification while antibiotics such as penicillin G, chloramphenicol, amphotericin and nalidixic acid do not affect the resolving potential of the reaction. This would be most important to sterile compounding pharmacies producing injectable antibiotic solutions. Method suitability should determine whether any antibiotics in the test sample could affect the assay.

In addition, RMTs or other rapid microbiological methods (RMMs) may be used as in-process controls prior to the final product release sterility test to provide faster information on the effectiveness of microbial controls and the early detection of gross contamination (enabling to investigate and restart production sooner) or probability that a product may fail sterility.

For the risk assessment, one consideration that may be overlooked is the relative risk to the patient based on the volume of the product injected or infused and the site of administration. The greater the volume and the more invasive, the greater the risk of blood stream infection for the patient. The risk ranges from a small volume of an intradermal injection to a large volume of an intravenous infusion (see *Table 1*).

Table 1. Typical Volumes by Route of Administration and the Relative Risk Level

Route of Administration	Typical Volume Administered (mL)	Risk Level
Intradermal	≤0.1	Very Low
Subcutaneous	≤1	Low
Intramuscular	≤3	Moderate
Intrathecal	1–10	High
Intravenous push	1–60	Moderate–High
Intravenous piggyback	25–250	High
Intravenous	>250	High

CRITICAL OPERATING PARAMETERS TO BE USED IN DETERMINING A RISK-BASED RAPID MICROBIAL TEST FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

The estimated operating parameters, i.e., LOD, time to results, and sample size, for the candidate technologies suitable for an RMT are found in *Table 2*.

Table 2. Operational Parameters of the Candidate Rapid Microbial Technologies

Candidate Technology	LOD (cfu)	Time to Result	Sample Size Range (mL)
Gram stain (for comparative purposes only)	10^4–10^5	30 min	0.1
⟨71⟩	Theoretical LOD of 1–3 cfu based on a Poisson distribution	14 days	40–500
Adenosine triphosphate (ATP) bioluminescence	1–10	2–7 days (including pre-enrichment)	1–1000
ATP bioluminescence	10^3	30 min	1–1000
Flow cytometry	10–100	6–8 h (pre-enrichment)	0.1–2
Isothermal micro-calorimetry	10^4	2–7 days	1
Nucleic acid-methods[a]	10–100	2–4 h	0.2–2
Respiration	1–10	Overnight to 7 days	Up to 10
Solid phase cytometry	1–10	2–3 h	1–1000

[a] For these methods, the signal would be in genomic units.

SITUATIONS WHEN ⟨71⟩ IS UNSUITABLE FOR PRODUCT RELEASE TESTING

Sample Size Consideration

The sample size tested may need to be reduced based on either the sample processing capability of the technology or the need to conserve the much-needed product.

The minimum quantity of product used for each medium and the minimum of units to be tested relative to batch size is found in ⟨71⟩, *Table 2* and *Table 3*. It is widely understood that for large pharmaceutical product batch sizes, the amount of units tested are not statistically based and has a reduced capability of detecting low contamination levels in individual product lots. However, with the smaller batch sizes of many short-lived products, this limitation will be reduced because the proportion of products tested relative to the batch size will increase.

This traditional sampling plan is not appropriate for a cell therapy product. For example, if 10 individual 60-mL IV bags of cells were prepared, then 4 bags would be sampled with 20 mL taken from each bag. With this sampling scheme 40% of the batch would be consumed for sterility testing representing not only a large economic loss, but also more importantly, a huge loss of therapeutic product that may prevent administration of a dose adequate to treat the patient. In contrast, if 40 individual 1-mL vials were consumed for the sterility testing of a 40,000-vial batch of an injectable drug product, this would represent only 0.1% of the batch.

Reducing the sample size and the number of units tested will reduce the sensitivity of the sterility test. *Table 3* and *Table 4* illustrate the relative insensitivity of the test for a pharmaceutical product and a CSP.

Table 3. The Probability That a 20-Unit Sterility Test Passes Given an Increasing Contamination Rate for a Drug Product

	Contaminated Items in the Batch (%)					
	0.1	**1**	**5**	**10**	**20**	**50**
p	0.001	0.01	0.05	0.1	0.2	0.5
q	0.999	0.99	0.95	0.9	0.8	0.5
Probability (*p*) of drawing 20 consecutive sterile items	0.98	0.82	0.36	0.12	0.012	<0.00001

Calculated from the following (*4*):

$$p = (1 - p)^{20} = q^{20}$$

p = proportion of contaminated containers in the batch
q = proportion of non-contaminated containers in the batch

Table 4. The Probability That a 6-Unit Sterility Test Passes Given an Increasing Contamination Rate for a CSP

	Contaminated Items in the Batch (%)			
	1	**5**	**10**	**20**
p	0.001	0.01	0.05	0.1

Table 4. The Probability That a 6-Unit Sterility Test Passes Given an Increasing Contamination Rate for a CSP *(continued)*

	Contaminated Items in the Batch (%)			
	1	**5**	**10**	**20**
q	0.999	0.99	0.95	0.9
Probability (*p*) of drawing 6 consecutive sterile items	0.995	0.94	0.73	0.53

Alternative sampling plans have been proposed in other compendia. One recommended approach to sterility testing of cell therapy products for a batch size of less than 40 units is found in *European Pharmacopoeia 9.0* chapter *2.6.27 Microbiological Examination of Cell-Based Preparations*.

The contamination test sample size for a cell preparation with a volume between 10 and 1000 mL would be 1% of the total volume; for a cell preparation with a volume between 1 and 10 mL, it would be a sample size of 0.1 mL; for a cell preparation less than 1 mL, the preparation would not be tested. As suggested in 21 CFR 610.12 (*Sterility*), in-process testing using rapid methods may be used in lieu of final product testing when fully justified.

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

Limit of Detection

Within the limitations of preparing inocula from a cell suspension with one or more colony-forming units, growth-based sterility tests can be shown to have at least a theoretical LOD of 1–3 cfu based on a Poisson distribution. Setting an LOD of a single viable cell with all technologies is an unrealistic barrier of entry for any sterility test, especially when the signal is not the colony-forming unit that is amplified by cultural enrichment.

Ability to Detect a Wide Range of Microorganisms

Although all the analytical platforms should have the ability to detect a wide range of bacteria, yeasts, and molds, it is of practical importance to demonstrate that technology chosen for an RMT is capable of detecting microorganisms implicated in sterility test failures, infection outbreaks, and product recalls associated with either CSPs, 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.

RAPID MICROBIAL TEST METHODS FOR THE RELEASE OF STERILE SHORT-LIFE PRODUCTS

Technologies recommended based on their match to the URS discussed above (*2*) suitable for an RMT are listed alphabetically as follows:

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

Brief Descriptions of the Technologies

Each of these candidate advanced analytical platforms is briefly discussed separately and key references are provided. For an overview, see Moldenhauer (*5*).

ADENOSINE TRIPHOSPHATE BIOLUMINESCENCE

This is a well-established technology with luminometers and reagents available from multiple instrument manufacturers. The energy from living cells is stored as ATP and 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 to 4 × 10^{-18} mole/cfu for gram-negative bacteria, 5 to 8 × 10^{-18} mole/cfu for gram-positive bacteria, and 300 to 800 × 10^{-18} mole/cfu for fungi (*6*). Given the high signal-to-noise ratio of the measurement and the background ATP in microbiological culture media, the microbiologically relevant instrument detection limit in broth is in the order of 5000 RLU equivalent to approximately 10^3 cfu.

This LOD will detect the presence of microorganisms at levels which are 3–4 logs less within an aliquot of the media than that is required for visual detection of growth in the media. For a rapid microbial test for the release of sterile short-life products, an enrichment culture either in liquid media to reach a threshold ATP level or on a membrane filter on solid media for the formation of microbial colonies 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 of 24–48 h (*7*). A labeling reagent consisting of either a fluorogenic substrate or vital stain is used to differentiate viable cells from dead cells and cellular debris. While bacteria are very small and may be hard to distinguish from cell debris, they can be differentiated by size, shape, and fluorescent intensity. Cell viability is indicated by the ability of the intact cell membrane to retain a fluorochrome generated by nonspecific cellular esterase, or by labeling the cell with nucleic acid-specific vital stains. A 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. The LOD for this technology may be >1 cfu and an enrichment step may be necessary.

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 and enrichment is needed for detection (2–7 days to result). Recently its application in pharmaceutical microbiology has been evaluated, although its specific application to release testing of sterile products has not been established yet (*8*).

NUCLEIC ACID AMPLIFICATION

Real-time quantitative polymerase chain reaction (PCR) has the potential to monitor the exponential phase of PCR through 36–48 cycles of amplification using universal primers and probes (termed "pan-bacterial" and "pan-fungal" methods) 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 is turned-over rapidly metabolically and would be a better indicator of viable microorganisms. For example, *Escherichia coli* contains 2 molecules of DNA and 2×10^4 molecules of 16S rRNA per cell (*9*). This process is achieved by the conversion of RNA into a complementary copy of DNA (cDNA) by the enzyme reverse transcriptase and the cDNA can be analyzed in real time in either a quantitative (enumeration test) or qualitative assay (sterility test). Alternatively, for DNA-based PCRs, a sample pretreatment with ethidium monoazide or propidium monoazide may also provide the capability to differentiate live from dead microbial cells (*10*,*11*), or free microbial DNA may be removed from a test sample by a centrifugation/washing step and the bacterial pellet used for analysis.

Realistically, an LOD of a single viable cell is probably an insurmountable challenge, especially for a test relying on a DNA/RNA target and universal primers. Another challenge is the differing amounts of genomic material in different microorganisms.

Generally, the LOD ranges from 10 to 1000 viable cells/mL in a sample and, in some reported cases, from 10 to 100 viable cells/mL. Recently it was shown that PCR may actually achieve detection of microorganisms with a limit of 10^2 to 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 (*12*). Adding a growth-based enrichment step for at least 24–48 h and comparing the PCR results before and after cultural enrichment may provide a practical solution for sterility testing. Alternatively, concentration methods could be applied to enrich the sample and reduce the sample volume. As noted by the authors of a recent study of the use of 16S rRNA 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*).

Direct comparison of growth-based and nucleic acid amplification-based assays is complicated by the fact that nucleic acid amplification-based assays also detect non-viable organisms and are a measure of microbial genome copy number, not colony-forming units. For that reason, the LOD for a nucleic acid amplification-based assay should be defined in terms of genome equivalents per milliliter.

Non-growth based RMTs for the release of sterile short-life products like nucleic acid amplification may have additional advantages due to the following:

- With close to real-time testing, the test will be completed before the short-life products are infused into a patient
- Detection of culture-negative infectious agents
- The test is minimally affected by antibiotics in the test sample, as indicated earlier in the chapter
- The test is less sensitive to background resulting from animal cell lysis (e.g., particles, ATP) as compared to other technologies since specific microbial genes are targeted

RESPIRATION

This broad category ranges from classical respirometers, to gaseous headspace analyzers to automated blood culture systems. Aerobic and anaerobic broth formulations allow for the recovery of most microorganisms responsible for blood stream infections within a 5-day incubation. With some instruments, this includes incubation at both 20°–25° and 30°–35° as in ⟨71⟩. This technology has been successfully extended to sterility testing of cell therapy products cells using a 7-day incubation as an alternative to the compendial sterility test for lot release (*14*).

Other instruments are available to detect and enumerate respiring microorganisms. For example, tunable diode laser absorption spectroscopy (TDLAS) can measure oxygen (O_2) depletion or carbon dioxide (CO_2) increase in closed units containing growing microorganisms in culture medium. The technology was originally developed to monitor gas headspace composition in closed units and also could be used for automatic media fill inspection (*8*,*15*). The system 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—All the systems of the respiration platform require microbial growth and metabolic activity for detection, i.e., the usual time of 2–7 days to obtain results is required. However, when the results may be progressively monitored to detect a sterility test failure earlier in the incubation period, that is a huge advantage with short-life products as explained in the risk assessment section above.]

SOLID PHASE CYTOMETRY

There are instrument systems based on solid phase cytometry combining fluorescent labeling and solid phase laser scanning to rapidly enumerate viable microorganisms in filterable liquids (*16*). Microorganisms are collected by filtration on 0.45-micron polyester membranes and treated with background and viability stains. The filters are scanned in a cytometer by a high-speed, 488 nM argon laser. Multiple photomultiplier tubes, processed to differentiate between labeled microorganisms and background noise, based on size, shape, and fluorescence intensity, detect fluorescence. The scan is displayed as a map that identifies the position of the fluorescent events that are verified using an epifluorescence microscope with an automated motorized stage to locate the individual events. The system is claimed to detect individual viable microorganisms in 2–3 h. It should be noted that most cell therapy products are non-filterable, so this technology may not be compatible with these types of products.

METHOD SUITABILITY TESTING

The method suitability requirements for a growth-based sterility test are given in ⟨71⟩. The suitability of the test must be demonstrated for each product to be tested. The recovery of the USP challenge organisms at a level less than 100 cfu, in the presence of residual product, is demonstrated as clearly visible growth in the microbiological growth media for either the direct inoculation or membrane filtration method. With signals other than the colony-forming unit derived from laboratory culture (e.g., nucleic acid, ATP, and fluorescent labeling of viable microbial cells), the results from testing actual samples may give results that are not equivalent to that using other technologies. However, method suitability testing will confirm that product residues in the analyte will not inhibit the enzymatic steps associated with the signal generation by the rapid method.

GLOSSARY

Colony-forming unit (cfu): Viable microorganisms capable of growth on solid microbiological culture media forming discrete, visible colonies.

Limit of detection (LOD): The lowest signal representing a viable microorganism that can be routinely detected.

Patient safety: Reduction of morbidity and mortality of the recipients of a contaminated product that is promoted by the completion of a rapid microbial test prior to the administration.

Positron emission tomography (PET): A nuclear medicine functional imaging technique that is used to observe metabolic processes in the body as an aid to the diagnosis of disease. The system detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body.

Risk-based rapid microbial test: A rapid microbial test selected by the stakeholder after a consideration of the LOD, sample size, specificity, and time to result that promotes patient safety by completing the test prior to the administration of a short-lived product to detect contamination, if any.

Specificity: Ability to detect a wide range of different bacteria, yeasts, and molds.

Time to result: Time to complete the microbial test and reach a conclusion about the lack of contamination of the sample test.

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