
Overview of United States and European Pharmacopoeia Chapters Dedicated to the Validation of Alternative Microbiological Methods

USP Chapter < 1223 > Validation of Alternative Microbiological Methods ***EP Chapter 5.1.6 Alternative Methods for Control of Microbiological Quality***

Introduction

These documents provide guidance on the selection and implementation of assay methodologies to serve as alternatives to compendial microbiological methods. The documents describe important steps that should be taken to evaluate candidate alternative methods, to select the analytical technology, and ultimately to qualify the method with actual product. These steps include, but are not limited to:

- 1.** Identification of a potentially suitable alternative methodology
- 2.** Demonstration that the method is equivalent and applicable as a replacement for a standard compendial method
- 3.** Development of user specifications for equipment selection
- 4.** Qualification of the method in the laboratory

In addition, the guidance outlines four distinct options for demonstrating equivalence (see *Demonstration of Equivalency* and [Table 2](#)).

Microbiological methods described in the compendia fall into two general categories:

- i.** *Qualitative methods* (not enumerative) that are used to assess the general microbial quality of compendial articles. This category includes assays that are intended to demonstrate the absence of microorganisms in a compendial article.
- ii.** *Quantitative methods* that yield a numerical (enumerative) result in terms of the microbial content of a compendial article.

There are inherent analytical factors that must be considered in the implementation of microbiological methods and in the comparison of a candidate alternative method to an existing compendial method. With respect to qualitative (“absence of”) analysis, it is critical to consider that in microbiology, the finding of “no microorganisms present” does not mean in absolute terms that zero cells are actually present. A result of “no growth” in a current compendial method is properly interpreted as “no growth was detected under the specified conditions”.

Limit of Detection and Differences in Cell Count Estimates between Growth-based Compendial Assays and Molecular Biochemical Alternative Analysis

The actual limits of detection of compendial microbiological methods have never been established quantitatively, and it is understood that many variables can affect the recovery of microorganisms. These variables include selection of growth media, incubation conditions, nutritional requirements of microorganisms that may be present, the physical condition of the microorganisms, and characteristics of the compendial article under test. Studies on the recovery of microorganisms from potable and environmental waters have demonstrated that traditional plate-count methods reporting cell count estimates as colony-forming units (cfus) may recover 0.1% to 1% of the actual microbial cells present in a sample, whereas alternative methods that use flow cytometry yield a different signal (cell count). The presence of a greater number of cells based on an alternative method with a signal other than cfu has not correlated with more user risk or a higher likelihood of pathogens being present when there is an established safety record. These results do indicate that in some types of samples, the mean estimated cell count recorded using a growth-based compendial assay may result in a very different mean value than a cell count estimate derived from a molecular biochemical analysis.

It is extremely important in the application of this chapter that users take into account that traditional growth-based microbiological methods constitute a logarithmic science. The inherent variability of these methods always must be considered in the selection, development, and validation of candidate alternative methods. The expectation of an unreasonable degree of agreement between modern molecular methods and traditional growth-based methods could complicate the implementation of newer analytical technologies.

Achieving the level of characterization (variability of the method) that is possible using modern chemical methods (e.g., high-performance liquid chromatography) is not possible in microbiology. Also, the enormous diversity of potential microorganisms in nature makes recovery of all types of microorganisms and their accurate enumeration or characterization an unrealistic target to achieve. The advent of modern molecular methods, which in some cases may recover higher cell counts than previously seen using existing compendial methods, should not be taken to mean that new risks exist that had not been heretofore recognized.

Validation Criteria

Traditional Validation Criteria (Qualitative Tests, e.g. sterility tests):

- **Specificity**
- **Limit of Detection**
- **Ruggedness**
- **Robustness**

Traditional Validation Criteria (Quantitative Tests, e.g. microbial enumeration):

- Accuracy
- Precision
- Specificity
- Limit of Detection
- Limit of Quantification
- Linearity
- Dynamic Range
- Ruggedness
- Robustness

What is Method Equivalency? Quotes and Comments from Regulatory Documents

USP General Notices 6.30: “Alternate methods may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction or in other special circumstances. Such alternate methods shall be validated as described in the General Informational USP Chapter *Validation of Compendial Procedures* < 1225 > and must be shown to give equivalent or better results.”

USP <1223>: “The critical question is whether or not the alternative method will yield results equivalent to, or better than the results generated by the conventional method.”

FDA Guidance *Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls Documentation*: “A validated alternative analytical procedure should be submitted only if is shown to perform equal to or better than the regulatory analytical procedure.”

ICH Q6A, 2.7 *Alternative Procedures*: “Alternative procedures are those which may be used to measure an attribute when such procedure control the quality of a drug substance or drug product to an extent that is comparable or superior to the official method.”

Equivalency was also fully discussed in the stimuli article Hauck, 2009 *Acceptable, equivalent or better approaches for alternatives to official compendial procedures*. Pharm. Forum. 35(3):772-778.

The concepts of acceptable procedures, performance, results and decision equivalency were introduced to the USP stakeholders in this reference.

User Requirements

Determining the precise requirements for an alternative microbiological method is essential before one can document the technology on suitability for use. This user requirements document should include all critical functions of the technology, critical user interface requirements, space requirements, operational requirements, and all other important characteristics of an alternative method for the intended use. These requirements will be specific to the company or organization, as well as to the alternative method's intended use, and therefore the requirements must be generated by the user.

In generating this user requirements specification (URS) document, the three separate components of the alternative microbiological method validation must be considered:

- 1. Instrument qualification.** Most alternative microbiological methods will depend on specific equipment. This analytical equipment is subject to industry standard instrument qualification requirements (see USP chapter < 1058 >, "Analytical Instrument Qualification" for further information).
- 2. Validation of alternate technologies.** The basic rationale for using an alternative methodology is to improve on some aspect of the existing technology of the current compendial method without sacrificing essential characteristics of that technology (e.g., plate count and membrane filtration). The current technology for compendial microbiology methods consists of detection of the growth of viable microorganisms on (or in) the nutrient medium. The alternative technology must be at least equivalent to the current technology in terms of performance for the intended use. Much of the technical support for equivalence may come from the peer-reviewed scientific literature or from a prior regulatory submission (e.g., a vendor submitted the Drug Master File to the FDA, or a company had a prior submission on this technology), but this must be confirmed as appropriate to the intended use.
- 3. Method suitability.** This consideration must address both the technology's suitability to the specific test and the lack of product inhibition and enhancement on the test results.
 - a. Suitability of the technology to the specific test.** Many compendial microbiological tests have mandated test requirements. An example of this would be sample plans consisting of the quantity of material to be tested (e.g., 10 g or 20 units of a specific volume). Because the test results are frequently used to determine compliance with finished product specifications, and the specifications are dependent on sample volume or quantity, the alternative technology must be able to satisfy sample volume requirements. The alternative technology is considered suitable if it can meet all critical parameters of the compendial test.
 - b. Inhibition and Enhancement.** Specific products may interfere or enhance the signal of different measurement technologies to the specific signal of interest (see USP chapter < 1227 >, "Validation of Microbial Recovery from Pharmacopoeial Articles"). This component of alternative microbiological method validation (i.e., suitability) must be demonstrated for each product tested.

Components of Data Quality

General USP information chapter < 1058 > describes four different components of data quality.

1. The most fundamental component is qualification of the instrument; that is, a demonstration that the instrument is functioning as designed.
2. Next in significance is the method validation: a demonstration that the technology is functioning as expected. For instance, this might be a demonstration that an alternative microbiological method is at least as suitable for its role in the test method as was the traditional plate count or recovery in nutrient broth.
3. Next in importance is the inclusion of relevant controls in the test to demonstrate the suitability of the test system.
4. The final component of data quality is the use of quality control check samples, a practice not commonly used in microbiology because analysts strive to exclude live cultures from a product testing area.

These different components of data quality are an important consideration in validating an alternative microbiological test as they help frame the URS.

Classical Microbiological Methods: The Concept of Colony Forming Units

The Colony Forming Unit (cfu) has been in use for about 125 years and continues to be specified as the unit of microbial enumeration in all USP monographs. However, it is important to understand that the cfu has always been an **estimation** of organisms present, rather than an actual count. The conceptualization of cfu as a signal requires a fundamental grasp of the process of plating bacteria or mold on solid media, as well as knowledge of what is required to produce a single colony.

The plate count method provides an estimate of the number of microorganisms present based on the growth of discrete colonies on an individual plate. Thus, the plate count is not a true cell count. Although it is theoretically possible for a single viable cell to give rise to a cfu, a single cell growing into a colony on a plate is unlikely to happen in nature. “Viability of a cell” is defined as the ability to multiply by binary fission such that a colony appears. For a colony to appear, viable cells must find specific conditions of nutrient growth medium, incubation, and time. Individual cells, however, are a rarity in nature, and it is far more likely that any colony growing on solid media arose from a clump, chain, or mass of cells deposited together. The cfu signal then is prone to underestimate the actual number of cells present in a sample. The extent of underestimation will vary, depending on the nature of the microorganism and the way in which the sample was prepared.

The cfu signal is also completely dependent upon growth, and if the nutritional or incubation conditions are not sufficient for the growth of colonies, the signal may be 0 cfu, or no growth, even when viable cells are present. Precision can be compromised further when organisms are present in large clumps, often associated with organic material, and are broken into smaller units during preparation. In this case, depending on the processing or handling of the sample, a clump could appear as a single colony or multiple colonies. Furthermore, the number of cfus on a plate must be in a countable range to have a statistically reliable enumeration.

Thus, the microbiological methods based on growth on agar plates represent a logarithmic science with a signal of enumeration (cfu) that is truly an estimate rather than a precise cell count. Understanding the strengths and weaknesses of the cfu as a signal is vital in the validation of an alternative method that uses an alternative signal. The cfu cannot be considered the only unit of microbiological enumeration, because it is only an estimate of cells present rather than an absolute measure.

Signals from Alternate Microbiological Methods

Rapid or modern microbiological methods may depend on other signals than cfu for microbial estimation and enumeration. These signals are often processed via instruments rather than visually. Extensive studies have been conducted on the capabilities of the various methods that can be applied to microbial assessment of compendial articles, and in most cases the prospective user will know the characteristics of the method and the signal it produces before selecting that method as an alternative. Guidance on method selection is provided in USP chapter <1223> Validation of Alternative Microbiological Methods, and in refereed scientific publications.

Most of the rapid microbiological methods are, to some extent, direct cell count methods. Therefore, they may provide a higher cell count estimate than the cfu method for a given sample, depending on how the method is used and which compendial article is under evaluation.

Some rapid methods detect and measure or enumerate cells on the basis of metabolic activity, which gives rise to a signal that can be measured instrumentally. Examples of these types of signals include adenosine triphosphate (ATP) content (bioluminescence), enzymatic activity, and changes to the composition of a nutritional broth or the headspace above the broth. It is possible that some cells may, due to their physiological condition, react in a manner that does not yield a detectable signal, or that they may be in a physiological state in which metabolism is limited. Recently, a new technology based on measuring oxygen consumption promises to be very sensitive and faster even in the presence of a very low microbial count (see **Figure 1**).

Alternative methods also may be based on vital staining, in which cells are stained or exhibit autofluorescence (based on cell component autofluorescence) and then are directly counted, either microscopically or instrumentally. To increase the probability that only living cells will be counted, multiple stains may be used, which can (1) increase sensitivity based on cell membrane function, (2) enhance reaction with nucleic acids, or (3) improve detection of metabolic activity.

There are also genetic analytical methods that can be used, as well as a range of other physicochemical methods of analysis that have been utilized in pharmaceutical, biopharmaceutical, clinical, and food microbiology. These methods may target, amplify, detect, or quantify a nucleic acid sequence, and it is important to understand the type of signal that results from the analytical method. In addition, one should understand the physiological characteristic of the microorganism that gives rise to the signal, which then makes it possible to enumerate the cells.



FIGURE 1. [SURCAPT™ MICROBIAL SURFACE DETECTION KIT](#)

Success Criteria

Alternative methods for obtaining a cell count may provide higher or lower cell counts than those provided by traditional compendial methods during the enumerative analysis of compendial articles. Higher cell counts are more commonly observed when using modern biochemical and instrumental methods compared to using traditional compendial methods that rely upon recovery and growth reported as cfu results. However, whether the cell counts are higher or lower with the alternative method, it is generally possible to correlate them with the estimates obtained using a compendial method.

Observations of cell counts that differ from cfu results are not a concern if the different methods and their different signals of cell presence are equivalent to or are non-inferior to reference methods in terms of assessing the microbiological safety of an article. Higher cell counts must not be considered as necessarily indicative of greater risk given the inherent variability of standard growth methods and the physical and chemical nature of compendial articles subject to analysis. This is especially true when enumerating microorganisms in articles that have a long history of safe and efficacious use. In such cases the discovery that an article contains a higher cell count than previously known does not mean that its safety has deteriorated.

Sample Size

Any alternative microbiological test method (within its intended purpose) must use a sample size and number of tests sufficient to produce an equivalent decision (or better) regarding microbiological quality as compared to the reference method.

Statistics and Alternative Methods

Attempts to use statistics to compare the cfu results to signals arising from biochemical, physiological, or genetic methods of analysis are probably of limited value. Given the differences among these methods, they cannot be expected to yield signals that could be compared statistically in terms of mean values and variability. Thus, the enumerative values, given as cfu results in association with reference methods, typically cannot be used as acceptance criteria for the assessment of articles via candidate alternative methods. Instead, it is the users' responsibility to propose values that they consider acceptable and unacceptable for the method that they have chosen. This will be done independently of existing standards expressed in terms of cfu.

Instrument Qualification

Instrument qualification should follow, at least in general terms, the discussion in 1058. The instrument qualification for equipment critical to the functioning of an alternative microbiological method involves four distinct phases:

1. Determination of the URS document
2. Installation qualification—Was the instrument installed correctly?
3. Operational qualification—Does the instrument meet the manufacturer’s specifications for correct operation?
4. Performance qualification—Does the instrument meet the URS for performance?

Validation of Alternative Technologies: User Requirements Specification

Preparation of the URS document should involve input from all stakeholders for the microbiological test method. These stakeholders may include representatives from the Microbiology, Quality, Regulatory Affairs, and Operations groups, as well as others. The time spent on this step should be considered an investment in reaching a clear understanding of the company’s needs before equipment is purchased, which will drive the performance qualification. At minimum, this document should include the following:

- Purpose and intended use (defined need for the instrument)
- Description of who will use the equipment
- Operational requirements (data format, user interfaces, and operating environment)
- Constraints (timetables, downtime, maintenance, user skill levels, product compatibility, limit of detection, accuracy, and rapidity)
- Life cycle (development, testing, delivery, validation, training, and obsolescence)
- Capability (turnaround time, test capacity and throughput, and labor requirements)
- Sustainability (consumables, calibration, validation, and preventative maintenance)

Refer to USP < 1058 > for information on the qualification of analytical instrumentation. The principles outlined in this chapter are generally applicable to the qualification of instruments used to conduct molecular biological or physiological alternative microbiological analysis. The user may need to tailor the specific recommendations in chapter < 1058 > to their particular instrument qualification specifications.

Validation Criteria

The validation parameters generally recommended for qualitative and quantitative microbiological tests are shown in **Table 1**. Examples of qualitative tests are the sterility test and the test for absence of specified microorganisms. A quantitative test would be microbial enumeration. Note that qualitative testing is binary, and for this reason there is generally no need to define equivalency of units of measure, only equivalency of outcome.

Table 1. Validation Parameters by Type of Microbiological Test		
VALIDATION PARAMETER	QUALITATIVE TESTS	QUANTITATIVE TESTS
Accuracy	No	Yes
Precision	No	Yes
Specificity	Yes	Yes
Limit of Detection	Yes	Yes
Limit of Quantification	No	Yes
Linearity	No	Yes
Operational (dynamic) range	No	Yes
Robustness	Yes	Yes
Repeatability	Yes	Yes
Ruggedness	Yes	Yes
Equivalency	Yes	Yes

Specificity

Definition

The specificity of an alternate qualitative microbiological method is defined as its ability to detect a range of challenge organisms specific to the technology. “Range of microorganisms” may be defined as a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are representative in terms of morphological and physiological attributes appropriate for the method and the product.

Demonstration

Specificity is demonstrated by comparable recovery of the challenge panel in both the compendial and alternate methods. The challenge is above the limit of detection or quantification but at a level that provides a measure of efficacy of the methods.

- 1. Growth based**—Add low numbers (around 100 cfu) of each microorganism on the panel and perform both the compendial and alternative methods to demonstrate recovery of the microorganism.
- 2. Nongrowth based**—Use suitable negative and positive controls to demonstrate that extraneous matter that may be in the system (e.g., extracellular ATP, DNA, or inhibition and enhancement factors) does not interfere with the detection of the defined range of microorganisms.

All microorganisms should be recovered and identified.

Limit of Detection

Definition

The limit of detection (LOD) of an alternate microbiological method is defined as the lowest number of microorganisms in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions. This should be conducted with the quality control organisms cited in USP Antimicrobial Effectiveness Testing < 51 >, Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests < 61 >, Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms < 62 >, Mycoplasma Tests < 63 > and Sterility Tests < 71 > as appropriate to the alternative method.

Demonstration

Method 1

- Inoculate with a serial dilution range of each challenge microorganism, appropriate for the intended use of the method and the technology. In most cases, the compendial media growth promotion test panel may be sufficient.
- The level of inoculation should be adjusted to a target of 50% of the dilution samples that show growth in the compendial test.
- Perform both the compendial and alternate tests.
- Tests should be repeated a sufficient number of times (statistically significant alpha risk: 0.05; beta risk: 0.20) for both the compendial and alternate tests.
- Statistics: Use the chi-square test or another appropriate approach to demonstrate equivalent recovery of the microorganism challenges.

Alternately, use Method 2.

Method 2 (MPN Method)

- Create a dilution series of the challenge organisms to include at least the range of 10 cfu to 10⁻² cfu (for a 10-fold series) or 5 cfu to 10⁻¹ cfu/inocula volume (for a 2-fold dilution series).
- Perform both the compendial and alternate tests with at least 5 simultaneous replicates of each dilution from the chosen series.
- Determine the most probable number (MPN) from three dilutions in the series that provide both positive and negative growth (or signal).

Ruggedness

Definition

The degree of precision of test results obtained by the analysis of the same samples under a variety of typical test conditions such as different analysts (3), instruments, and reagent lots (the method for demonstration may follow instrument or materials supplier recommendations, or it could be based solely on data supplied by test method manufacturer).

Robustness

Definition

The user may rely on data supplied by test method manufacturer.

A measure of robustness is not a comparison between the compendial and alternate methods. Rather, it is a necessary component of validation of the alternate method so that the user understands the limits of the operating parameters of the method.

Method Suitability

For each new product to be tested using the validated alternate microbiological method, perform the suitability test as described in general test methods (see chapters < 51 >, < 61 >, < 62 >, < 63 >, and < 71 >), using the number of units and quantities prescribed, the sample preparation appropriate for the product, and the required test sensitivity to determine the absence of a product effect that would obscure the signal of the method.

Method suitability may be demonstrated using three independent tests. Only the accuracy and precision validation parameters are required for quantitative methods and the accuracy parameter for qualitative methods.

After an alternative method has been shown to be equivalent to the compendial test with one product, it is not necessary to repeat the equivalency parameter for every new product. It is merely necessary to verify the method. For example, with each new product, one must demonstrate that residual product does not interfere with the concentration, extraction, purification, and recovery of the target nucleic acid, or the polymerase chain reaction (PCR) amplification and chemical probe detection of the target ribosomal ribonucleic acid (rRNA) gene sequence.

Equivalency

All microbiological tests are performed to enable informed decision making regarding the microbiological quality of a product, raw material, component, or process step. In this respect, the intended purpose of microbiological tests may be to either evaluate for the presence or absence of microorganisms (as in the sterility test), or to estimate the number of organisms present. The technological means by which microbiological test methods assess microbiological quality and enable a product-quality decision may differ from the growth-based means

typical of reference methods. The units of measurement (signal) of a microbiological quality assessment performed using alternative microbiological test methods will generally not be a cfu, but rather a different approach to obtaining a cell count estimate. Therefore, the validation of alternative microbial methods should involve two components: (1) equivalence demonstration, and (2) analytical method and equipment qualification.

Equivalence Demonstration

General Notices and Requirements states that alternate methods may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction. It further stipulates that alternative methods can be implemented in other special circumstances. Such alternate methods shall be validated as described in < 1225 > and must be shown to produce equivalent or better results than the referee method for any given quality attribute. When comparing two test procedures to show equivalent or better performance, statistical evidence is assembled to show equivalence or, in statistical terms, noninferiority. For example, with microbial enumeration, equivalency may be shown if there is no statistically significant difference between the two means generated when enumerating with the compendial and alternative methods. However, this may not be possible when the two methods yield different signals. Examples of this situation are when the microbial enumeration method uses vital staining of microbial cells or measurement of genomic material in place of cfus.

Similarly, the FDA *Guidance for Industry* document *Analytical Procedures and Methods Validation: Chemistry, Manufacturing, and Controls Documentation* states that a validated alternative analytical procedure should be submitted only if it is shown to have performance equal to or better than the regulatory analytical procedure. Also, section 2.7 of the ICH document *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Q6A)* states that alternative procedures are those that may be used to measure an attribute when such a procedure controls the quality of a drug substance or drug product to an extent that is comparable to or superior to the official method. However, other options to demonstrate equivalence are available and are discussed in *Demonstration of Equivalency*.

Demonstration of Equivalency

Four options are available to establish the equivalence of a candidate alternative analytical method:

- **Option 1: Acceptable Procedures** (i.e., merely meeting a minimum performance or acceptance requirement)
- **Option 2: Performance Equivalence of Equivalent or Better**
- **Option 3: Results Equivalence**
- **Option 4: Decision Equivalence** ([1](#))

A comparison of these four equivalence options is given in **Table 2**. The multiple equivalence options reflect the diversity in the technology and applications of the alternative test methodologies.

Table 2. Equivalence Option Matrix

OPTION	DEMONSTRATION	COMPARISON TO OFFICIAL COMPENDIAL METHOD	BASED ON NUMERICAL RESULTS OR CONCLUSIONS	NUMBER OF CHARACTERISTICS
1. Acceptable Procedures	Acceptable	No	Results	Multiple
2. Performance Equivalence	Equivalent or Better	Yes	Results	Multiple
3. Results Equivalence	Equivalent	Yes	Results	Single
4. Decision Equivalence	Equivalent	Yes	Conclusions	Single

Option 1: Acceptable Procedures

When this option is used, no direct comparison between the candidate alternative method and an official compendial method is required. Instead, a reference material with known properties may be used, such as a standard inoculum of a specific microorganism, a quantity of highly purified bacterial genome, or another appropriate signal. In some cases, it could be required that the alternative method measures the signal in the presence of the test sample, with validation criteria that are consistent with the capability of the technology, as described in the scientific literature.

Option 2: Performance Equivalence

Performance equivalence requires the demonstration of equivalent or better results with respect to validation criteria—such as accuracy, precision, specificity, limit of detection, limit of qualification, robustness, and ruggedness—that may be appropriate for the intended use of the alternative qualitative or quantitative method. It is possible that the alternative method may be worse at one or more of the listed test functions compared with the official method and still be acceptable because of the advantages of the alternative method. This may be the case if the alternative method has any of the advantages stated in the *General Notices and Requirements* (methods “may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances”). Other special circumstances would include improvements in time to result or the cost of running the test. If a candidate alternative method is suitable for assessing the quality of the material tested, it may still be acceptable, even if it differs from the official method in one or more test functions. The final analytical qualification criteria should reflect only the criteria that the microbiologist deems necessary to achieve performance equivalence.

Option 3: Results Equivalence

When results equivalence are required, the hypothesis to be tested is that the alternative and compendial test methods give equivalent numerical results. This contrasts with the evaluation of the validation parameters, as is done in performance equivalence. Because the same sample cannot be tested in microbiology, typically a tolerance interval is established when comparing the two methods, with the alternative method determined to be numerically superior or noninferior. Reports on the use of alternative nongrowth-based methods have shown that

they may produce significantly higher cell count estimates than a growth method that reports outcomes in cfu. In this case, the analyst could use a calibration curve showing a correlation between the two methods in the product specification range.

Option 4: Decision Equivalence

A decision equivalence is similar to a results equivalence but differs in that a numerical result is not generated. Instead a pass/fail result is obtained. With this approach, the frequency of positive and negative results generated should be no worse than with the compendial method. For the purposes of qualification, laboratory studies involving spiking low levels of microorganisms may be considered. The following sections provide suggested approaches for demonstrating that the alternative procedure is equivalent to or better than the compendial procedure. Other approaches may be used with justification.

Equivalence Demonstration for Alternative Qualitative Microbiological Procedures

Results obtained by procedures in USP chapters < 62 >, < 63 >, and , < 71 > are indicative of the presence or absence of microorganisms. These tests do provide a decision (i.e., the compendial article either passes or fails the test). This type of data fits in the decision equivalence category as described in the Stimuli article ([1](#)). Approach 1 is based on demonstrating decision equivalence. *Approach 2: Compare MPN Results* is an alternative that converts the qualitative results to quantitative ones by using the most probable number (MPN) procedure. Both approaches use a noninferiority hypothesis ([2](#)).

To demonstrate the acceptability of the alternate procedure relative to the current microbiological procedure, the laboratory must demonstrate that the new procedure is equivalent to or better than the current procedure in terms of sensitivity for detecting the presence of organisms. In general, a recommended approach for comparing the alternate procedure to the compendial procedure is to use a noninferiority test (one-sided, as in noninferiority clinical trials for new drug products) ([3](#)) rather than two-sided equivalence [as in bioequivalence ([4](#))]. Noninferiority is an appropriate approach for two reasons. First, from a patient perspective, it is beneficial to promote an alternative procedure that is more sensitive than the referee procedure. In contrast, a two-sided approach penalizes better sensitivity. Note, though, that companies will need to assess the risk associated with the change in procedure, because a more sensitive procedure may generate more positive results. Second, the new procedure has benefits (principally, reduced time to a result) that make it preferable to the microbiological procedure, even if it is not as sensitive, as long as it is close enough.

Approach 1: Use Present and Absent Results

The noninferiority hypothesis for this approach is that the proportion of samples that produce a signal for the new procedure (P_N) is NMT some amount ($\delta > 0$) less than the proportion for the current, compendial procedure (P_C) ([5](#)):

$$\text{Result} = P_N - P_C \geq -\delta$$

The δ is the noninferiority margin. Unless the laboratory requires a tighter margin, use $\delta = 0.20$ in the experiments described below. Calculate a two-sided 90% confidence interval for $P_N - P_C$ ([6](#)). Noninferiority is concluded if the lower confidence limit exceeds -0.20 . If the experiment is able to conclude in favor of the noninferiority hypothesis, then it can be stated, with 95% confidence, that $P_N \geq P_C - 0.20$ at the bioburden level studied.

This evaluation needs to be conducted using multiple types of microorganisms to represent the range of microorganisms encountered. The choices can follow < 71 > or appropriate suitability test organisms, organisms recovered from product failure, or problematic organisms that would be analytically challenging to the alternative test procedure.

The laboratory should conduct an evaluation to determine whether the alternate procedure can be shown to be noninferior to the microbiological procedure in terms of sensitivity as measured by the proportion of samples growing colonies. For each organism in a qualitative test, conduct three evaluations. The first uses samples at or around 1 cfu, where no growth is likely to be observed (hence no signal will be detected by the growth-based microbiological procedure) to characterize the sensitivity of the new procedure at this level. The second uses samples at or around 100 cfu, where the microbiological procedure would be expected to detect a relatively high percentage of the signal, to determine the acceptability of the new procedure. The third is a comparison of the two procedures at a burden where 50% –75% of samples would be expected to grow colonies (often around 10 cfu) to test the noninferiority hypothesis as described above. In the noninferiority experiment for qualitative microbiology tests, a minimum of 75–100 samples should be tested on each procedure. Using 75 samples provides approximately 80% power, whereas using 100 samples provides approximately 90% power, both for concluding noninferiority if the two procedures are actually equally sensitive using $\delta = 0.20$. If the laboratory believes their new procedure is less sensitive, a larger number of samples is required to maintain these power levels.

Independent samples: Suppose that N_A samples have been tested with the candidate alternative procedure, of which X_A samples are positive, and that N_C samples (not the same as those tested with the candidate) have been tested with the compendial procedure and that X_C samples are positive. Calculate the following:

$$\hat{p}_A = X_A / N_A \quad \hat{p}_C = X_C / N_C$$

$$\theta = N_C / N_A$$

$$a = 1 + \theta$$

$$b = -[R(1 + \theta\hat{p}_C) + \theta + \hat{p}_A]$$

$$c = R(\hat{p}_A + \theta\hat{p}_C)$$

$$\tilde{p}_A = [-b - (b^2 - 4ac)^{1/2}] / (2a)$$

$$\tilde{p}_C = \tilde{p}_A / R$$

$$V = \frac{\tilde{p}_A(1 - \tilde{p}_A)}{N_A} + R^2 \frac{\tilde{p}_C(1 - \tilde{p}_C)}{N_C}$$

$$Z = \frac{(\hat{p}_A - R\hat{p}_C)}{\sqrt{V}}$$

Conclude noninferiority if $Z > z_\alpha$ where z_α is the upper α percentage point of a standard normal distribution.

Paired samples: Suppose that N samples have been tested by both the candidate alternative and compendial procedures. The results can be displayed in a 2×2 table (see **Table 3**).

Table 3. Results for Paired Samples			
COMPENDIAL PROCEDURE			
ALTERNATIVE PROCEDURE	POSITIVE	NEGATIVE	ROW TOTALS
Positive	X_{11}	X_{10}	X_A
Negative	X_{01}	X_{00}	$N - X_A$
Column Totals	X_C	$N - X_C$	N

Compute the following:

$$L = [X_{10} - RX_{01} + (1 - R)X_{11}] / N$$

$$V = X_A(X_{10} + X_{01}) / X_C^3$$

$$Z = L / \sqrt{V}$$

Conclude noninferiority if $Z > z_\alpha$ where z_α is the upper α percentage point of a standard normal distribution.

Approach 2: Compare MPN Results

For both the compendial reference and the alternative procedures, conduct an MPN comparative study using standard procedures for MPN for each of the N samples per procedure. Ideally, the same samples are used for the two procedures, but this is not a necessity.

For Approach 2, the noninferiority hypothesis is:

$$\mu_A - \mu_C \geq \log(R) \text{ or } \text{antilog}(\mu_A - \mu_C) \geq R$$

where μ_A and μ_C are the means in the log scale for the alternative and compendial procedures, respectively.

Independent samples: Determine MPN for N_A samples by the alternative procedure, convert all values to logs, and determine the sample mean of the log values (\bar{x}_A) and sample variance of the log values (S_A^2). Similarly, determine \bar{x}_C and S_C^2 from the logs of N_C samples tested with the compendial procedure. Determine the following:

$$L_{low} = \bar{X}_A - \bar{X}_C - t_{\alpha, df} \sqrt{\frac{S_A^2}{N_A} + \frac{S_C^2}{N_C}}$$

where t_α, df is the upper α percentage point of the t distribution with df degrees of freedom and

$$df = \frac{(S_A^2/N_A + S_C^2/N_C)^2}{\frac{(S_A^2/N_A)^2}{N_A - 1} + \frac{(S_C^2/N_C)^2}{N_C - 1}}$$

If using software that only allows for integer degrees of freedom (e.g., Excel®), use linear interpolation to obtain the t value. Conclude noninferiority if $\text{antilog}(L_{low}) \geq R$.

Paired data: Determine MPN for N samples by the alternative procedure and for the same N samples by the compendial procedure, convert all $2N$ values to logs, and determine the sample mean (\bar{x}) and variance (S^2) of the differences of log alternative value minus log compendial value. Determine the following:

$$L_{low} = \bar{x} - t_{\alpha, N-1} S / \sqrt{N}$$

where $t, N - 1$ is the upper α percentage point of the t distribution with $N - 1$ degrees of freedom. Conclude noninferiority if $\text{antilog}(L_{low}) \geq R$.

Equivalence Demonstration for Alternative Quantitative Microbiological Procedures

A key characteristic of potential alternative quantitative procedures is that their signal will differ from the cfu of the compendial microbiological procedure. As a consequence, equivalence as it is typically considered cannot be shown; that is, the numerical results are expected to differ in magnitude and units. Instead, this chapter proposes two criteria for candidate alternative quantitative procedures:

1. Results from the candidate procedure have at least acceptable precision (repeatability).
2. The results from the candidate procedure are highly correlated with those from the compendial procedure. A high correlation is taken to indicate that quantitative acceptance criteria expressed in cfu can be calibrated to criteria in the unitage of the alternative procedure.

Precision

Prepare a minimum of six samples at a minimum of two bioburden levels near specification limits relevant to the laboratory. Run the candidate procedure for the prepared samples. [NOTE — This is to correspond to repeatability conditions; see <1225>.] At each level, determine the sample variance (S^2) of the logarithms of sample results. Calculate the following:

$$UL = 100 * \left[\text{anti log} \left(\sqrt{\frac{(n-1)S^2}{\chi_{.05, n-1}^2}} \right) - 1 \right]$$

Where n is the number of samples ($n \geq 6$) and $\chi_{.05, n-1}$ is the lower 5% value of a chi-square distribution with $n - 1$ degrees of freedom. Precision is acceptable if $*UL \leq \sigma$, where σ is the predetermined maximal acceptable repeatability percent geometric coefficient of variation, %GCV. (For small values, the %GCV will be approximately the %RSD.)

The greater the number of samples (n) the greater the likelihood (power, in statistical terms) that a procedure, the precision of which is actually acceptable, will yield data that meet this criterion and thus be declared acceptable. The lab may use prior data to determine a value of n that meets their needs.

Example

For the alternative procedure data in **Table 4**, calculate the following:

$$n = 10$$

$$S^2 = 0.000241, \text{ and}$$

$$\chi_{.05, 9}^2$$

$$= 3.325113, \text{ so}$$

$$UL = 6.06\%$$

This alternative procedure thus has acceptable repeatability precision as long as the prespecified σ had been at least 6.06%.

Correlation

Prepare a minimum of two samples at each of four different bioburden levels covering the range from near limit of qualification (LOQ) to one log above the specification limit. Determine activity for all samples by both the candidate alternative and compendial procedures. Plot and determine the correlation between the log of values from the candidate alternative procedure (y) and the log of values from the compendial procedure (x). The correlation is acceptable if at least 0.95 (or R^2 at least 0.9025).

Although a linear relationship between the two sets of results is typically expected, a nonlinear relationship is acceptable. In the case of a nonlinear relationship, use the Spearman (nonparametric) correlation instead of the Pearson correlation.

Table 4. Compendial and Alternative Data	
COMPENDIAL (CFU)	ALTERNATIVE (CELL COUNT)
70	970
71	965
75	950
92	990
100	1000
105	1051
116	1046
123	1039
127	985
130	1020

Figure 2 shows the plot of these data after conversion to base 10 logs. Because R^2 does not meet the stated requirement, the results from these two procedures are not sufficiently correlated.

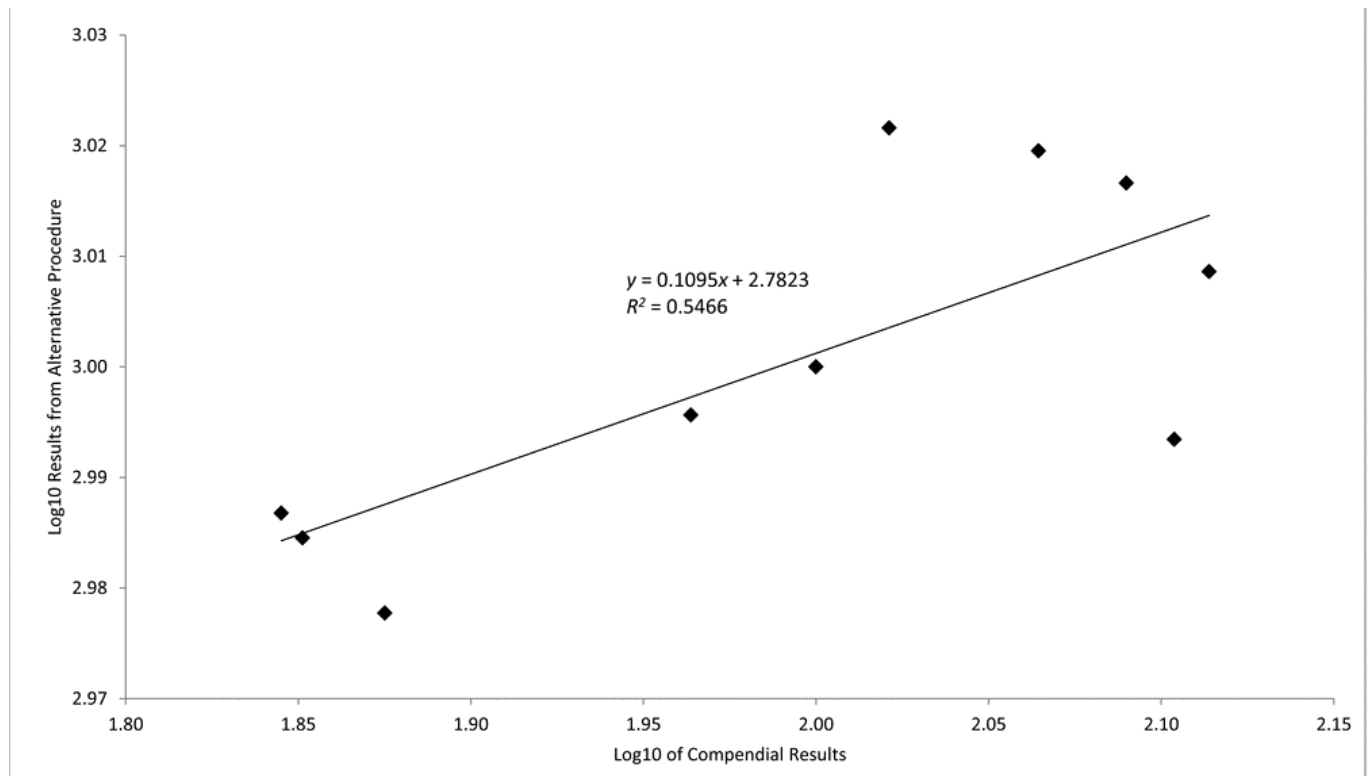


FIGURE 2. LOG10 RESULTS - COMPENDIAL VS. ALTERNATIVE

Candidate alternative procedures that may be suitable for making decisions about the microbiological quality of a sample (as in **Figure 2**) may not correlate well enough with the compendial procedure to meet the above correlation requirement. In that case, the other option is to apply the decision equivalence approach as described earlier for qualitative tests. During procedure development, the lab determines a candidate cut point to correspond to the compendial cut point for the required level of microbiological quality. For example, if the required level of microbiological quality is NMT 10^2 cfu, for which the compendial cut point is 200 cfu for a positive result, the laboratory will need to determine an acceptance criterion for the candidate alternative procedure that will essentially match that value. Then, the validation experiment to confirm this choice proceeds as described earlier for qualitative tests.

Statistical Tools

As with process analytical chemistry, new developments in microbiological measurements make real-time bioburden monitoring using signals other than cfus in air, water, in-process materials, and finished products possible. For example, in a pharmaceutical water system, parameters including flow rate, total organic carbon, conductivity, pH, total particulates, bacterial endotoxin, and viable bacteria as autofluorescent particles could be monitored in real time and subjected to multivariate analysis. Any cfus will be replaced by other signals, such as autofluorescent particles, in a particular size range that may give higher counts but can be correlated using multivariate calibrations. In addition, multivariate statistical tools are available for process understanding, multivariate statistical process control, out-of-limit detection, and general inference of product quality. These tools include multivariate control charting, principal component analysis, and multiway partial least square analysis (7,8).

GLOSSARY

Accuracy: Closeness of the test results obtained by the alternative test method to the value obtained by the compendial method, to be demonstrated across the range of the method.

Alternative microbiological method: A modern or rapid microbiological test procedure (MMM or RMM) that is different from the traditional growth-based method, such as the plate count or recovery in liquid broth. The alternative or rapid method may use instrumentation and software to manage the testing and analyses of data and may provide quantitative (enumeration) or qualitative (detection) microbial identification.

Colony-forming unit (cfu): An estimate of the number of microorganisms obtained by traditional plate count methods. Because it is uncertain whether a colony was derived from the growth of one or even one thousand cells, the results are reported as cfu/mL (for a liquid) or cfu/g (for a solid) and not as cells/mL or cells/g.

Conventional microbiological method: A classical or traditional growth-based method, such as enumeration on an agar plate or detection in a liquid broth when incubated for a specified time and temperature. These methods are used in < 51 > , < 61 > , < 62 > , < 63 > , and < 71 > .

Equivalence: When the test results from two procedures are sufficiently close for the intended use of the procedures. Demonstration of equivalence requires a prespecified measure of how similar the test results need to be.

False negative: A test result that is incorrectly determined as negative (e.g., the absence of a viable microbial detection result when viable microorganisms are present). A type II error, also known as an error of the second kind, occurs when the null hypothesis is false but erroneously fails to be rejected. It is failing to assert what is, in fact, present—a miss. A type II error may be compared with a so-called false negative in a test (and seen as a “miss”) that is checking for a single condition with a definitive result of true or false. The rate of the type II error is denoted by the Greek letter β and is related to the power of a test (which equals $1 - \beta$).

False positive: A test result that is incorrectly determined as positive (e.g., a viable microbial detection result when viable microorganisms are not present). In statistical test theory, the idea of a statistical error is an integral part of hypothesis testing. These are described as type I and type II errors. A type I error, also known as an error of the first kind, occurs when the null hypothesis (H_0) is true but is rejected. It is asserting something exists that is, in fact, absent (i.e., a false hit). A type I error may be compared with a so-called false positive (a result that indicates that a given condition is present when it actually is not present) in tests where a single condition is tested. The rate of the type I error is called the “size” of the test and denoted by the Greek letter α . It usually equals the significance level of a test. In the case of a simple null hypothesis, α is the probability of a type I error.

Independent samples: Samples selected from the same population or different populations that have no effect on one another. That is, no correlation exists between the samples.

Limit of detection (LOD): The lowest concentration of microorganisms in a test sample that can be detected, but not necessarily quantified, under defined experimental conditions.

Limit of quantification (LOQ): The lowest number of microorganisms in a test sample that can be enumerated with acceptable accuracy and precision under defined experimental conditions.

Linearity: The ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range.

Method suitability: Demonstration of lack of a negative or positive influence of the product on the signal generated by the method.

Multivariate analysis: A set of techniques dedicated to the analysis of data sets with more than one variable. These techniques include multiple linear regression (MLR), where several independent variables (which are supposed to be fixed or equivalently are measured without error) are used to predict, with a least square approach, one dependent variable.

Multiway partial least square analysis: Methods that take into account the ordered way in which data are collected, usually organized into time-ordered blocks that are representative of a multiple sample or process run. Multiway, partial least-square analysis can use data from statistical process-control charts using both process data and product quality control data, allowing determination of how the variance of the process data is predictive of the product quality.

Noninferiority: Demonstration that the alternate method is not worse than the compendial method by more than a small prespecified amount. This amount is known as the noninferiority margin or δ .

Paired samples: A sample of matched pairs of similar units.

Principal component analysis: A technique used to express variations of many variables by a small number of indices. It is used for data compression and information extraction, finding the combination of variables or factors that describe major trends in a data set. Because there is a great deal of correlated or redundant information in process measurements, essential information related to a process may not lie in any individual process variable but in how variables change with respect to one another.

Range: The interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with specified accuracy, precision, and linearity.

Repeatability precision: The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of the same suspension of microorganisms and uses different suspensions across the range of the test. Also known as “repeatability”.

Robustness: A method’s capacity to remain unaffected by small but deliberate variations in method parameters, providing an indication of its reliability during normal usage.

Ruggedness: Intermediate (within laboratory) precision associated with changes in operating conditions. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay (e.g., different days, different analysts, different equipment).

Specificity: The ability to detect a range of microorganisms, which demonstrate that the method is fit for its intended use.

Validation: The process of demonstrating and documenting that the performance characteristics of a procedure and its underlying method meet the requirements for the intended application and that the procedure is thereby suitable for its intended use. Formal validations are conducted prospectively according to a written plan that includes justifiable acceptance criteria on validation procedures.

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Addendum

Validation Parameters for Studies Comparing Current (Conventional) Method vs a Qualitative RMM

Table A.1	
PARAMETER	ACCEPTANCE CRITERIA
Specificity	1. All required microbial cultures must be detected by the test system. 2. All other components in the sample do not interfere with the test.
Limit of Detection	Lowest number of microorganisms in a sample that can be detected under the stated experimental conditions.
Ruggedness	The resistance to influences of operational and environmental variables (different analysts, instruments, reagent lots, and laboratories)
Robustness	The system capacity to remain unaffected by small but deliberate variations in method parameters. It provides an indication of system reliability during normal usage.
Repeatability	All replicates inoculated with challenge organisms must be determined to be positive.

Table A.2		
PARAMETER	INTERPRETATION	REQUIREMENT/ACCEPTANCE CRITERIA
Specificity	Ability to detect a range of microorganisms that may be present in the test article. Also, the assurance that extraneous matter does not interfere with the test.	<ol style="list-style-type: none"> 1. Use microorganisms appropriate for the purpose of the alternative method. 2. All required microorganisms must be detected by the new method. 3. All other components in the sample do not interfere with the test. 4. Quantitative: Challenge RMM at low counts (< 50 CFU) to check for false positives.
Limit of Detection	The lowest number of microorganisms in the original sample that can be detected.	<ol style="list-style-type: none"> 1. Run at least 5 replicates as close to 1 cfu as possible, or at least NMT 5 cfu. 2. Adjust inoculum until at least 50% of the samples show growth. 3. Analyze by Chi Square test. 4. Quantitative: Use Most Probable Number. A 5-tube design in a tenfold dilution series could be used for both methods. These would then be challenged with equivalent inoculums (for example, a 10^{-1}, 10^{-2}, and 10^{-3} dilution from a stock suspension of approximately 50 cfu per mL to yield target inocula of 5, 0.5, and 0.05 cfu per tube, respectively) and the MPN of the original stock determined by each method. If the 95% confidence intervals overlapped, then the methods would be considered equivalent. 5. Alternative method should be able to provide results at least as low as traditional methods.

Table A.2		
PARAMETER	INTERPRETATION	REQUIREMENT/ACCEPTANCE CRITERIA
Ruggedness	<p>The degree of precision of test results obtained by analysis of the same sample under a variety of normal test conditions.</p> <p>This parameter is not required as part of the EP guidance.</p>	<p>It is the degree of precision of test results obtained by analysis of the same samples under a variety of normal test conditions, such as:</p> <ol style="list-style-type: none"> 1. Different analysts 2. Different instruments 3. Different reagent lots 4. Different laboratories
Robustness	<p>A measure of the test's capacity to remain unaffected by small, deliberate variations in method parameters and provides an indication of its reliability during normal use.</p>	<p>Check small changes in temperature, humidity, or any other relevant method parameter.</p>
Repeatability	<p>Per USP < 1223 ></p> <p>A measure of the test's ability to reproduce results within a degree of confidence using the same instrument and same laboratory over a short period of time.</p> <p>This parameter is not required as part of the EP guidance.</p>	<p>Not specified in the Quantitative or Qualitative section of this USP Chapter.</p>

Table A.3	
PARAMETER	ACCEPTANCE CRITERIA
Accuracy	Closeness of the new method test results to the value obtained by the traditional method.
Precision	Degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of microbial suspensions or different lots of same product across the range of the test.
Range	The interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with precision, accuracy, and linearity.
Limit of Quantification	The lowest number of microorganisms that can be accurately counted.
Linearity	Ability to produce results that are proportional to the number of microorganisms present in the sample within a given range.
Equivalence	Equivalence of the new method to the pharmacopoeia method.

Table A.4		
PARAMETER	INTERPRETATION	REQUIREMENT/ACCEPTANCE CRITERIA
Accuracy	Closeness of the test results obtained by the alternate test method to the value obtained by traditional methods. Demonstrated across the range of results.	<ol style="list-style-type: none"> 1. At least 5 suspensions across the range of the test should be analyzed for each challenge organism. 2. Recovery of not less than 70% of recovered microorganisms of the estimate provided by the traditional method should be achieved 3. Or, the new method should be shown to recover at least as many microorganisms as the traditional method by appropriate statistical analysis. 4. The operational range of both methods should overlap.
Precision	Degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of suspensions of laboratory microorganisms across the range of the test.	<ol style="list-style-type: none"> 1. At least 5 suspensions across the range of the test should be analyzed. For each suspension at least 10 replicates should be assayed in order to be able to calculate statistically significant estimates of the standard deviation or relative standard deviation (coefficient of variation). Generally, a RSD in the 15% to 35% range would be acceptable. 2. Irrespective of the specific results, the alternate method should have a coefficient of variation that is not larger than that of the traditional method.

Table A.4		
PARAMETER	INTERPRETATION	REQUIREMENT/ACCEPTANCE CRITERIA
Operational Range	Interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with precision, accuracy, and linearity.	This parameter will be estimated from the experimental parameter studies of precision, accuracy, and linearity.
Limit of Quantification	Lowest number of microorganisms that can be accurately counted.	<ol style="list-style-type: none"> 1. Run at least 5 replicates across the range of the measurement. 2. Due to the inherent nature of bacterial enumeration and the Poisson distribution of bacterial counts, the alternative method need only demonstrate that it is at least as sensitive as the traditional method to similar lower limits.
Linearity	The ability to produce results that are proportional to the concentration of microorganisms present in the sample.	Run at least 5 different concentrations over the range of measurement. Run at least 5 replicates at each. R2 may be used, should not be < 0.95.
Equivalence	The under revision chapter defines that there is generally no need to define equivalency of units of measure, only equivalency of outcome.	Use Performance Equivalence (See below).

Demonstration of Equivalency (from currently under revision < 1223 >)

Four options are available to establish the equivalence of a candidate alternative analytical method: (1) acceptable procedures (i.e., merely meeting a minimum performance or acceptance requirement); (2) performance equivalence of equivalent or better; (3) results equivalence; and (4) decision equivalence ([1](#)). A comparison of these four equivalence options is given in **Table A.5**. The multiple equivalence options reflect the diversity in the technology and applications of the alternative test methodologies.

Table A.5 Equivalence Option Matrix				
OPTION	DEMONSTRATION	COMPARISON TO OFFICIAL COMPENDIAL METHOD	BASED ON NUMERICAL RESULTS OR CONCLUSIONS	NUMBER OF CHARACTERISTICS
1. Acceptable Procedures	Acceptable	No	Results	Multiple
2. Performance Equivalence	Equivalent or Better	Yes	Results	Multiple
3. Results Equivalence	Equivalent	Yes	Results	Single
4. Decision Equivalence	Equivalent	Yes	Conclusions	Single

Option 1: Acceptable Procedures

When this option is used, no direct comparison between the candidate alternative method and an official compendial method is required. Instead, a reference material with known properties may be used, such as a standard inoculum of a specific microorganism, a quantity of highly purified bacterial genome, or another appropriate signal. In some cases, it could be required that the alternative method measure the signal in the presence of the test sample, with validation criteria that are consistent with the capability of the technology, as described in the scientific literature.

Option 2: Performance Equivalence

Performance equivalence requires the demonstration of equivalent or better results with respect to validation criteria—such as accuracy, precision, specificity, limit of detection, limit of qualification, robustness, and ruggedness—that may be appropriate for the intended use of the alternative qualitative or quantitative method. It is possible that the alternative method may be worse at one or more of the listed test functions compared with the official method and still be acceptable because of the advantages of the alternative method. This may be the case if the alternative method has any of the advantages stated in the *General Notices and Requirements* (methods “may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances”). Other special circumstances would include improvements in time to result or the cost of running the test. If a candidate alternative method is suitable for assessing the quality of the material tested, it may be still acceptable, even if it differs from the official method in one or more test functions. The final analytical qualification criteria should reflect only the criteria that the microbiologist deems necessary to achieve performance equivalence.

Option 3: Results Equivalence

When results equivalence is required, the hypothesis to be tested is that the alternative and compendial test methods give equivalent numerical results. This contrasts with the evaluation of the validation parameters, as is done in performance equivalence. Because the same sample cannot be tested in microbiology, typically a tolerance interval is established when comparing the two methods, with the alternative method determined to be

numerically superior or noninferior. Reports on the use of alternative nongrowth-based methods have shown that they may produce significantly higher cell count estimates than a growth method that reports outcomes in cfu. In this case, the analyst could use a calibration curve showing a correlation between the two methods in the product specification range.

Option 4: Decision Equivalence

A decision equivalence is similar to a results equivalence but differs in that a numerical result is not generated; instead, a pass/fail result is obtained. With this approach, the frequency of positive and negative results generated should be no worse than with the compendial method. For the purposes of qualification, laboratory studies involving spiking low levels of microorganisms may be considered. The following sections provide suggested approaches for demonstrating that the alternative procedure is equivalent to or better than the compendial procedure. Other approaches may be used with justification.

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