

《 1223 》 VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

INTRODUCTION

The purpose of this chapter is to provide guidance for validating methods for use as alternatives to the official compendial microbiological methods. For microbial recovery and identification, microbiological testing laboratories sometimes use alternative test methods to those described in the general chapters for a variety of reasons, such as economics, throughput, and convenience. Validation of these methods is required. Some guidance on validation of the use of alternate methods is provided in the *Tests and Assays* section in the *General Notices and Requirements*. This section also notes that in the event of a dispute, only the result obtained by the compendial test is conclusive.

Validation studies of alternate microbiological methods should take a large degree of variability into account. When conducting microbiological testing by conventional plate count, for example, one frequently encounters a range of results that is broader (%RSD 15 to 35) than ranges in commonly used chemical assays (%RSD 1 to 3). Many conventional microbiological methods are subject to sampling error, dilution error, plating error, incubation error, and operator error.

[Validation of Compendial Procedures](#) 《 1225 》 defines characteristics such as accuracy, precision, specificity, detection limit, quantification limit, linearity, range, ruggedness, and robustness in their application to analytical methods. These definitions are less appropriate for alternate microbiological method validation as “at least equivalent to the compendial method” given the comparative nature of the question (see the *Tests and Assays—Procedures* section in *General Notices and Requirements*). The critical question is whether or not the alternate method will yield results equivalent to, or better than, the results generated by the conventional method.

Other industry organizations have provided guidance for the validation of alternate microbiological methods.* The suitability of a new or modified method should be demonstrated in a comparison study between the USP compendial method and the alternate method. The characteristics defined in this chapter may be used to establish this comparison.

TYPES OF MICROBIOLOGICAL TESTS

It is critical to the validation effort to identify the portion of the test addressed by an alternate technology. For example, there is a variety of technologies available to detect the presence of viable cells. These techniques may have application in a variety of tests (e.g., bioburden, sterility test) but may not, in fact, replace the critical aspects of the test entirely. For example, a sterility test by membrane filtration may be performed according

to the compendial procedure up to the point of combining the processed filter with the recovery media, and after that the presence of viable cells might then be demonstrated by use of some of the available technologies. Validation of this application would, therefore, require validation of the recovery system employed rather than the entire test.

There are three major types of determinations specific to microbiological tests. These include tests to determine whether microorganisms are present in a sample, tests to quantify the number of microorganisms (or to enumerate a specific subpopulation of the sample), and tests designed to identify microorganisms. This chapter does not address microbial identification.

Qualitative Tests for the Presence or Absence of Microorganisms

This type of test is characterized by the use of turbidity in a liquid growth medium as evidence of the presence of viable microorganisms in the test sample. The most common example of this test is the sterility test. Other examples of this type of testing are those tests designed to evaluate the presence or absence of a particular type of microorganism in a sample (e.g., coliforms in potable water and *E. coli* in oral dosage forms).

Quantitative Tests for Microorganisms

The plate count method is the most common example of this class of tests used to estimate the number of viable microorganisms present in a sample. The membrane filtration and Most Probable Number (MPN) multiple-tube methods are other examples of these tests. The latter was developed as a means to estimate the number of viable microorganisms present in a sample not amenable to direct plating or membrane filtration.

General Concerns

Validation of a microbiological method is the process by which it is experimentally established that the performance characteristics of the method meet the requirements for the intended application, in comparison to the traditional method. For example, it may not be necessary to fully validate the equivalence of a new quantitative method for use in the antimicrobial efficacy test by comparative studies, as the critical comparison is between the new method of enumeration and the plate count method (the current method for enumeration). As quantitative tests, by their nature, yield numerical data, they allow for the use of parametric statistical techniques. In contrast, qualitative microbial assays, e.g., the sterility test in the example above, may require analysis by nonparametric statistical methods. The validation of analytical methods for chemical assays follows well-established parameters as described in [Validation of Compendial Procedures](#) { 1225 }. Validation of microbiological methods shares some of the same concerns, although consideration must be given to the unique nature of microbiological assays (see [Table 1](#)).

Table 1. Validation Parameters by Type of Microbiological Test

Parameter	Qualitative Tests	Quantitative Tests
Accuracy	No	Yes
Precision	No	Yes
Specificity	Yes	Yes
Detection limit	Yes	Yes
Quantification limit	No	Yes
Linearity	No	Yes
Operational range	No	Yes
Robustness	Yes	Yes
Repeatability	Yes	Yes
Ruggedness	Yes	Yes

VALIDATION OF QUALITATIVE TESTS FOR DEMONSTRATION OF VIABLE MICROORGANISMS IN A SAMPLE

Specificity

The specificity of an alternate qualitative microbiological method is its ability to detect a range of microorganisms that may be present in the test article. This concern is adequately addressed by growth promotion of the media for qualitative methods that rely upon growth to demonstrate presence or absence of microorganisms. However, for those methods that do not require growth as an indicator of microbial presence, the specificity of the assay for microbes assures that extraneous matter in the test system does not interfere with the test.

Limit of Detection

The limit of detection is the lowest number of microorganisms in a sample that can be detected under the stated experimental conditions. A microbiological limit test determines the presence or absence of microorganisms, e.g., absence of *Salmonella* spp. in 10 g. Due to the nature of microbiology, the limit of detection refers to the number of organisms present in the original sample before any dilution or incubation steps; it does not refer to the number of organisms present at the point of assay.

One method to demonstrate the limit of detection for a quantitative assay would be to evaluate the two methods (alternative and compendial) by inoculation with a low number of challenge microorganisms (not more than 5 cfu per unit) followed by a measurement of recovery. The level of inoculation should be adjusted until at least 50% of the samples show growth in the compendial test. It is necessary to repeat this determination several times, as the limit of detection of an assay is determined from a number of replicates

(not less than 5). The ability of the two methods to detect the presence of low numbers of microorganisms can be demonstrated using the Chi square test. A second method to demonstrate equivalence between the two quantitative methods could be through the use of the Most Probable Number technique. In this method, a 5-tube design in a ten-fold 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) and the MPN of the original stock determined by each method. If the 95% confidence intervals overlapped, then the methods would be considered equivalent.

Ruggedness

The ruggedness of a qualitative microbiological method is the degree of precision of test results obtained by analysis of the same samples under a variety of normal test conditions, such as different analysts, instruments, reagent lots, and laboratories. Ruggedness can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the microbiological method. Ruggedness is a validation parameter best suited to determination by the supplier of the test method who has easy access to multiple instruments and batches of components.

Robustness

The robustness of a qualitative microbiological method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. Robustness is a validation parameter best suited to determination by the supplier of the test method. As there are no agreed upon standards for current methods, acceptance criteria are problematic and must be tailored to the specific technique. It is essential, however, that an estimate of the ruggedness of the alternate procedure be developed. The measure of robustness is not necessarily a comparison between the alternate method and the traditional, but rather a necessary component of validation of the alternate method so that the user knows the operating parameters of the method.

VALIDATION OF QUANTITATIVE ESTIMATION OF VIABLE MICROORGANISMS IN A SAMPLE

As colony-forming units follow a Poisson distribution, the use of statistical tools appropriate to the Poisson rather than those used to analyze normal distributions is encouraged. If the user is more comfortable using tools geared towards normally distributed data, the use of a data transformation is frequently useful. Two techniques are available and convenient for microbiological data. Raw counts can be transformed to normally distributed data either by taking the \log_{10} unit value for that count, or by taking the square root of count +1. The latter transformation is especially helpful if the data

contain zero counts.

Accuracy

The accuracy of this type of microbiological method is the closeness of the test results obtained by the alternate test method to the value obtained by the traditional method. It should be demonstrated across the operational range of the test. Accuracy is usually expressed as the percentage of recovery of microorganisms by the assay method.

Accuracy in a quantitative microbiological test may be shown by preparing a suspension of microorganisms at the upper end of the range of the test, that has been serially diluted down to the lower end of the range of the test. The operational range of the alternate method should overlap that of the traditional method. For example, if the alternate method is meant to replace the traditional plate count method for viable counts, then a reasonable range might be from 10^0 to 10^6 cfu per mL. At least 5 suspensions across the range of the test should be analyzed for each challenge organism. The alternate method should provide an estimate of viable microorganisms not less than 70% of the estimate provided by the traditional method, or the new method should be shown to recover at least as many organisms as the traditional method by appropriate statistical analysis, an example being an ANOVA analysis of the \log_{10} unit transforms of the data points. Note that the possibility exists that an alternate method may recover an apparent higher number of microorganisms if it is not dependent on the growth of the microorganisms to form colonies or develop turbidity. This is determined in the *Specificity* evaluation.

Precision

The precision of a quantitative microbiological method is the 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. The precision of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). However, other appropriate measures may be applied.

One method to demonstrate precision uses a suspension of microorganisms at the upper end of the range of the test that has been serially diluted down to the lower end of the range of the test. 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. Irrespective of the specific results, the alternate method should have a coefficient of variation that is not larger than that of the traditional method. For example, a plate count method might have the RSD ranges as shown in the following table.

Table 2. Expected RSD as a Function of cfu per Plate

cfu per Plate	Expected RSD
30 – 300	<15%
10 – 30	<25%
<10	<35%

Specificity

The specificity of a quantitative microbiological method is its ability to detect a panel of microorganisms suitable to demonstrate that the method is fit for its intended purpose. This is demonstrated using the organisms appropriate for the purpose of the alternate method. It is important to challenge the alternate technology in a manner that would encourage false positive results (specific to that alternate technology) to demonstrate the suitability of the alternate method in comparison to the traditional method. This is especially important with those alternate methods that do not require growth for microbial enumeration (for example, any that do not require enrichment or can enumerate microorganisms into the range of 1 – 50 cells).

Limit of Quantification

The limit of quantification is the lowest number of microorganisms that can be accurately counted. As it is not possible to obtain a reliable sample containing a known number of microorganisms, it is essential that the limit of quantification of an assay is determined from a number of replicates ($n > 5$) at each of at least 5 different points across the operational range of the assay. The limit of quantification should not be a number greater than that of the traditional method. Note that this may have an inherent limit due to the nature of bacterial enumeration and the Poisson distribution of bacterial counts (see [Validation of Microbial Recovery from Pharmacopeial Articles](#) { 1227 }). Therefore, the alternate method need only demonstrate that it is at least as sensitive as the traditional method to similar lower limits.

Linearity

The linearity of a quantitative microbiological test is its ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range. The linearity should be determined over the range of the test. A method to determine this would be to select at least 5 concentrations of each standard challenge microorganism and conduct at least 5 replicate readings of each concentration. An appropriate measure would be to calculate the square of the correlation coefficient, r^2 , from a linear regression analysis of the data generated above. While the correlation coefficient does not provide an estimate of linearity, it is a convenient and commonly applied measure to approximate the relationship. The alternate method should not have an r^2 value less than 0.95.

Limit of Detection

See *Limit of Detection* under *Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample*.

Range

The operational range of a quantitative microbiological method is the interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with precision, accuracy, and linearity.

Ruggedness

See *Ruggedness* under *Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample*.

Robustness

See *Robustness* under *Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample*.

* PDA Technical Report No. 33. The Evaluation, Validation and Implementation of New Microbiological Testing Methods. *PDA Journal of Pharmaceutical Science & Technology*. 54 Supplement TR#33 (3) 2000 and Official Methods Programs of AOAC International.

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