



Standard Guide for Evaluating Non-culture Microbiological Tests¹

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1. Scope

1.1 The purpose of this guide is to assist users and producers of non-culture microbiological tests in determining the applicability of the test for processing different types of samples and evaluating the accuracy of the results. Culture test procedures such as the Heterotrophic (Standard) Plate Count, the Most Probable Number (MPN) method and the Spread Plate Count are widely cited and accepted for the enumeration of microorganisms. However, these methods have their limitations, such as performance time. Moreover, any given culture test method typically recovers only a portion of the total viable microbes present in a sample. It is these limitations that have recently led to the marketing of a variety of non-culture procedures, test kits and instruments.

1.2 Culture test methods estimate microbial population densities based on the ability of microorganisms in a sample to proliferate in or on a specified growth medium, under specified growth conditions. Non-culture test methods attempt to provide the same or complimentary information through the measurement of a different parameter. This guide is designed to assist investigators in assessing the accuracy and precision of non-culture methods intended for the determination of microbial population densities or activities.

1.3 It is recognized that the Heterotrophic Plate Count (HPC) does not recover all microorganisms present in a product or a system (**1, 2**).² When this problem occurs during the characterization of a microbiological population, alternative standard enumeration procedures may be necessary, as in the case of sulfate-reducing bacteria. At other times, chemical methods that measure the rates of appearance of metabolic derivatives, the utilization of contaminated product components or genetic profile of the microbial population might be indicated. In evaluating non-culture tests, it is possible that the use of these alternative standard procedures might be the only

means available for establishing correlation. In such cases, this guide can serve as a reference for those considerations.

1.4 Because there are so many types of tests that could be considered non-culture based, it is impossible to recommend a specific test protocol with statistical analyses for evaluating the tests. Instead, this guide should assist in determining what types of tests should be considered to verify the utility and identify the limitations of the nonconventional test.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

2. Referenced Documents

2.1 *ASTM Standards*:³

D1129 Terminology Relating to Water

D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water

D5245 Practice for Cleaning Laboratory Glassware, Plasticware, and Equipment Used in Microbiological Analyses

D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1601 Practice for Conducting an Interlaboratory Study to Evaluate the Performance of an Analytical Method

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

3.1 *Definitions*:

3.1.1 For definitions of terms used in this guide refer to Terminologies **D1129**, **E2756**, and **E177**.

3.2 *Abbreviations*:

3.2.1 **HPC**—Heterotrophic Plate Count

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² The boldface numbers in parentheses refer to the list of references at the end of this guide.

³ For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

4. Summary of Guide

4.1 ASTM standard methods and practices are referenced for use by producers and users in order to determine the potential utility of a non-standard, non-culture test.

4.2 Recognizing that potential users of non-culture test methods might not have the resources with which or capabilities for evaluating the utility of non-standard, non-culture test methods, recommendations are provided to assist those users in identifying the capabilities that qualify microbiological laboratories to perform collaborative studies to evaluate those methods.

5. Significance and Use

5.1 This guide should be used by producers and potential producers of non-culture tests to determine the accuracy, selectivity, specificity, and precision of the tests, as defined in Practice E691. Results of such studies should identify the limitations and indicate the utility or applicability of the non-culture test, or both, for use on different types of samples.

5.2 Non-culture test users and potential users should employ this guide to evaluate results of the non-culture test as compared to their present methods. Practices D5245 and D5465 should be reviewed in regards to the microbiological methods employed. If culture methods have not been used for monitoring the systems, then guidelines are included for obtaining microbiological expertise.

5.3 Utilization of a non-culture test can reduce the time required to determine the microbiological status of the system and detect microbe that are not detected by culture testing. Consequently, non-culture tests can contribute to the improvement in the overall operating efficiency of microbial contamination condition monitoring and diagnostic efforts, and micro-bicide performance evaluations.

5.4 Detecting microbial contamination levels that exceed predetermined upper control limits indicates the need for an addition of an antimicrobial agent or other corrective maintenance action. By accurately determining this in a shorter time period than is possible than by culture methods, treatment with antimicrobial agents may circumvent more serious problems than if the treatment were postponed until culture results were

available. If the antimicrobial treatment program relied on an inaccurate non-culture test, then unnecessary loss of product and problems associated with inappropriate selection or improper dosing with antimicrobial agents would exist.

5.5 Since many methods based on entirely different chemical and microbiological principles are considered, it is not possible to establish a unique design and recommend a specific method of statistical analyses for the comparisons to be made. It is only possible to present guides that should be followed while performing the experiments. It is also recommended that a statistician be involved in the study.

6. Procedures

6.1 Practice E1601 provides guidance on the evaluation of analytical method performance. The guidance provided below amplifies the processes described in Practice E1601 as they apply to microbiological test methods.

6.2 Although the heterotrophic plate count (HPC) has been used historically to determine the utility of newly developed non-culture methods, and can be an appropriate reference method in many cases (3), there are cases for which HPC is not an appropriate reference method

6.2.1 The choice of referee method to use for validating a new or proposed non-culture method should be determined based on the parameter the new method purports to be measuring.

6.2.2 Several methods used for the HPC are listed in Table 1.

6.2.3 When none of the Table 1 variations of the HPC (Heterotrophic Plate Count) are suitable reference methods, Adenosine Triphosphate Concentration (Test Method D4012) or the Most Probable Number (MPN) technique (7) may be more appropriate.

6.2.4 Alternative standard enumeration methods or methods for measuring the rate of the appearance of derivatives or the rate of disappearance of components of the product in which the microbial contamination is being measured—where such phenomena are known to be correlated to microbial contamination levels—may also be used as referee methods for assessing the accuracy and precision of a novel non-culture method.

TABLE 1 Comparison of Selected Heterotrophic Plate Count Procedures for Samples from Various Sources

	Water (4)	Dairy (5)	Environment (6)	Food (7)	Cosmetic (7)	Paper (8)	Pharmaceutical (9)
Media	TGE, SM, R2A or m-HPC	SM	SM or TGE	SM	ML	TGE	SCD
Dilution, H ₂ O	KH ₂ PO ₄ + MgCl ₂	KH ₂ PO ₄	KH ₂ PO ₄	KH ₂ PO ₄	MLB	H ₂ O	KH ₂ PO ₄
Incubation, °C	35 ± 0.5 20 or 28 (R2A)	32 ± 1	35 ± 0.5	35	30 ± 2	36 ± 0.5	30–35
Incubation, h	48 ± 3 72 ± 4 (bottled water) 72–168 (R2A medium)	48 ± 3	48	48 ± 2	48	48	48–72
Amount of Agar, mL	10–12 (Pour Plate) 15 (Spread Plates) 5 (Membrane Filter)	10–12	10+	12–15	Spread Plates	15–20	15–20

TGE = Tryptone Glucose Extract Agar
 SM = Standard Methods Agar (Tryptone Glucose Yeast Agar)
 ML = Modified Lethen Agar
 MLB = Modified Lethen Broth
 SCD = Soybean Casein Digest Agar
 R2A = Low-Nutrient Media (which may not be available in dehydrated form)
 m-HPC = Formerly called m-SPC Agar (used for membrane filtration)

6.2.5 No single method is universally applicable; consequently, it is imperative to determine the rationale for employing any given measurement procedure and to select a standard that will permit the determination of whether or not the method achieves the objectives defined in the scope of the procedure.

6.3 A knowledge of standard microbiological technique is required in order to conduct microbiological test method evaluations. If that expertise is not currently available in-house, consult an outside testing laboratory.

6.3.1 Many industrial microbiology laboratories are certified for the analysis of drinking water by the EPA or the state government, or both (a listing of these laboratories can be obtained from the regional EPA office or the state government).

6.3.2 These and other independent microbiology laboratories often specialize in processing samples from different industries

6.3.3 Suitable microbiology laboratories are typically often listed as “Laboratories—Testing” in the telephone book or in directories such as the ASTM International Directory of Testing Laboratories³. It is important that this document be referenced when undertaking an evaluation with an outside laboratory.

6.4 For each method, first list of all known major sources of variability.

6.4.1 For example, major sources of variability can include:

6.4.1.1 *Sample heterogeneity*—non-uniform distribution of physical (for example: temperature and viscosity), chemical (for example: layering caused by eutrophication) and microbiological (for example: population density, taxonomic diversity and physiological state of microbes).

6.4.1.2 *Sample perishability*—changes in taxonomic profile (diversity and relative abundance of individual taxa contained in sample).

6.4.1.3 Storage and handling conditions.

6.4.2 Measures must be taken to minimize the individual and net contributions of these factors when evaluating test method precision.

6.4.3 When designing a non-culture test method evaluation, ensure that the microbial bioburdens in the samples cover the new method’s expected quantification range. Minimally the test plan shall include three samples (test levels) of each test matrix for which the candidate method is expected to be appropriate:

- Low bioburden* – microbial contamination just above the method’s expected lower limit of quantification
- Medium bioburden* – microbial contamination in the mid-range of the method’s detection range
- High bioburden* – microbial contamination near the upper limits of the method’s detection range

6.4.3.1 For the purposes of this practice, each bioburden range is a test *level*. Thus the levels must cover the range of interest for each intended application.

6.4.3.2 A test *matrix* is the type material in which the microbes are found (for example: water, industrial fluids, soils, coatings, etc.)

6.5 At each test level, analyze replicate samples, by both the method being evaluated, and by the standard or reference method. The number of replicates depends on the number of sources of variability. Thus, in the previous-mentioned example of non-culture test (6.4.2), it is necessary to analyze at least two replicate samples at each level (preferably more) by both the reference and candidate method.

6.5.1 The standard or reference method used will often be one of the methods listed in Table 1, however, in matrices from which culture test results are likely to be inaccurate or suspected of being inaccurate, data from the candidate method can be compared with data from non-microbiological parameters known to covary with bioburden.

6.6 A suitable test plan is shown in Table 2.

6.6.1 In this example, at each level, three replicates are analyzed by the non-culture, candidate method and by the HPC method. These numbers of replicates will vary according to the method.

6.6.2 Although Practice E1601 prescribes a minimum of duplicate tests per analyst/laboratory, a minimum of three replicates substantially improves the robustness of the method validation effort.

6.6.3 A full interlaboratory study requires at least 30 degrees of freedom, including participation of no fewer than six laboratories and a sufficient range of samples to address the issues outlined in 6.4. See Table 2 and Practice E691.

6.6.4 For initial test method robustness evaluations it is sufficient to have two participants (either individual analysts or different laboratories) so that preliminary repeatability and reproducibility estimates can be computed.

TABLE 2 Test Plan for Evaluating Candidate Non-culture test Methods

Candidate Method	Test Level ^A	Analyst/Lab	Replicate test	Reference Method ^B	Replicate test
1	1 (low)	1	1	HPC	1
			2		2
			3		3
		2	1		1
			2		2
			3		3
	2 (medium)	1	1	HPC	1
			2		2
			3		3
		2	1		1
			2		2
			3		3
	3 (high)	1	1	HPC	1
			2		2
			3		3
		2	1		1
			2		2
			3		3
Total Number of Tests			18		18

^A Test plans shall include a minimum of three levels of the test parameter per sample: one with bioburden just above the candidate method’s lower limit of quantification, one in the mid-range and with a high bioburden. The objective is to test precision across the candidate method’s quantification range. The test plan shall also include at least two samples in order to meet the minimum 30 degrees of freedom requirement.

^B Although this example uses HPC as the reference method, other methods can be more appropriate for a given evaluation (5.1).

6.6.5 Although the correlation between the candidate test parameter and bioburden can be determined from data produced by replicate testing of three samples, the reliability of correlation statistics increases with the number of samples tested. A minimum of five samples is appropriate for establishing the relationship between test method results and bioburden.

6.6.5.1 In order to minimize the impact of uncontrollable variables, it is most appropriate to dilute a high bioburden sample in the test matrix to produce a sample set that includes a range of bioburdens.

6.6.5.2 The appropriate dilution factor will depend on the type of data produced by the candidate test method. Typically 2- fold, 5-fold and 10-fold extinction dilution series are appropriate.

6.6.5.3 In an extinction dilution series, the most dilute sample will have a bioburden that is below the candidate test method’s lower limit of detection.

6.7 Inclusion of a standard or reference method in a new method’s evaluation plan is not mandatory. However it serves an educational purpose by providing a bases for assessing the relative bias between the new method and the reference method.

6.7.1 There are no reference standards with which to determine the true bias of any microbiological test method. Consequently, it is impossible to determine the bias of either a standard or candidate method, but important to investigate the relative bias of the new method relative to traditional methods

6.7.2 To illustrate this point, consider the relative bias among a culture method, a direct count method and a chemical method.

- Direct count data typically have a positive bias relative to culture data.

- Chemical data also typically have a positive bias relative to culture data.

- Chemical data typically have a negative bias relative to direct count data.

6.7.3 Relative bias among alternative microbiological test methods can be attributed to individual or multiple factors including but not limited to:

- *Differential impact of interferences* – chemicals that interfere with one method but not another.

- *Heterogeneity* – generally, the larger the sample size, the smaller the impact of non-uniform biomass distribution.

- *Sample preparation* – for example: inadequate disaggregation of bacterial flocs contribute to HPC underestimation of the culturable biomass, but is less likely to affect chemical concentration test data (protein, ATP, etc.).

- *Systemic error* – if methods being compared are consistently

run in the same order, time-related issues rather than factors inherent in either method can cause apparent bias.

6.8 Practice E1601 provides detailed instructions for computing repeatability, reproducibility, and bias.

7. Report

7.1 Guidance provided in Practice E1601 should be used to report the results of a new method evaluation study.

7.1.1 A description of the test method(s) and test plan shall be provided.

7.1.2 Evaluation study participants shall be identified. Pseudonyms or codes can be used to preserve participant confidentiality.

7.1.3 Test results shall be provided in table form.

7.1.3.1 Typically participants are listed down the first column and samples are listed across the first row, as illustrated in Table 3:

7.1.4 Compute means (\bar{x}) and standard deviations (s) for each set of replicates and record these values in a second table. This table will the differences (d) between (\bar{x}) for each replicate set and the grand mean ($\bar{\bar{x}}$) for the total data set, s^2 and d^2 as illustrated in Table 4:

7.1.5 Use equations provided in Practice E1601 to compute the method’s standard deviation, the repeatability standard deviation and the reproducibility standard deviation.

7.1.6 If only the candidate method has been included in the evaluation, plot mean test results as a function of dilution factor.

7.1.6.1 If appropriate (for example, test results are spread across several orders of magnitude) transform raw data into appropriate units (such as $\text{Log}_{10} X$, where X is the test result) before plotting data.

7.1.6.2 Compute the regression equation and correlation coefficient between test data and dilution factor.

NOTE 1—Simple linear regression computations, such as those available within most commercial spreadsheet software, are not appropriate for analyzing data obtained per Table 3. A mixed effects regression model such as the one outlined in Practice D4012 can be fit to these data. Such a regression model assigns random effect for participant and a fixed effect for test level.

7.1.7 If two or more parameters have been included in the evaluation, plot each candidate method as a function of the reference method.

7.1.7.1 Compute the regression equation as described in 7.1.6.1 and 7.1.6.2.

7.2 Under certain circumstances, when the relationship between two parameters is constant, the standard deviations

TABLE 3 Sample Test Data Table

Analyst/Lab Number	Sample		
	A	B	C
1	X _{A11}	X _{B11}	X _{C11}
	X _{A12}	X _{B12}	X _{C12}
	X _{A13}	X _{B13}	X _{C13}
2	X _{A21}	X _{B21}	X _{C21}
	X _{A22}	X _{B22}	X _{C22}
	X _{A23}	X _{B23}	X _{C23}

Where X is the test result for sample A, B, or C; analyst/laboratory 1 or 2, and replicate, 1, 2, or 3, respectively.

TABLE 4 Statistical Computations for Candidate Test Method

Analyst/Lab #	Test Results							
	A	B	C	\bar{X}	s	d	s ²	d ²
1	\bar{X}_{A1}	\bar{X}_{B1}	\bar{X}_{C1}	\bar{X}_1	s ₁	$\bar{X}_1 - \bar{X}$	(s ₁) ²	$(\bar{X}_1 - \bar{X})^2$
2	\bar{X}_{A2}	\bar{X}_{B2}	\bar{X}_{C2}	\bar{X}_2	s ₂	$\bar{X}_2 - \bar{X}$	(s ₂) ²	$(\bar{X}_2 - \bar{X})^2$
				\bar{X}			$\sum s^2$	$\sum d^2$

obtained by the new method can be converted, by appropriate statistical procedures, into equivalent units of the standard/reference method by using the calibration line for conversion, 7.1.

7.3 Different parameters reflect different properties of the test population. For example, the concentration of adenosine triphosphate is nominally 1 fg/cell, but can vary between 0.1 and 20 fg/cell depending on the taxa present and the respective physiological states of those taxa. Consequently, caution must be exercised when using values of one microbiological parameter to determine the values of a second parameter by calculation.

7.4 In view of the complexity of the problem and variety of situations that can arise, it is not possible to recommend additional procedures and statistical methods, or both. A more detailed discussion of statistical methods may be found in the *Statistical Manual of the Association of Official Analytical Chemists* (10) and in Chapter 14, “The Comparison of Method of Measurements,” of *The Statistical Analysis of Experimental Data* (11).

8. Keywords

8.1 bacteria; correlation; culture; enumeration; microbiology; non-culture methods

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