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Standard Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria[1]

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1. Scope

1.1 The purpose of this guide is to assist users and producers of nonconventional tests in determining the applicability of the test for processing different types of samples and evaluating the accuracy of the results. Conventional procedures such as the Heterotrophic (Standard) Plate Count, the Most Probable Number (MPN) method and the Spread Plate are widely cited and accepted for the enumeration of microorganisms. However, these methods have their limitations, such as performance time and degree of accuracy. It is these limitations that have recently led to the marketing of a variety of non-conventional procedures, test kits and instruments.

1.2 A conventional test is one that is widely accepted and published as a standard microbiological method or related procedure. A new, nonconventional test method will attempt to provide the same information through the measurement of a different parameter. This guide is designed for comparing levels of bacteria recovered from samples by the Heterotrophic Plate Count Procedure to the equivalent units determined with a nonconventional test.

1.3 It is recognized that the Heterotrophic Plate Count does not recover all microorganisms present in a product or a system[2] **(1)**. When this problem occurs during the characterization of a microbiological population, alternate 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 or the utilization of contaminated product components might be indicated. In evaluating nonconventional tests, the use of these alternate standard procedures may be the only means available for establishing correlation. In such cases, this guide can serve as a reference for those considerations.

1.4 Since there are so many types of tests that could be considered nonconventional, 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.

2. Referenced Documents

2.1 *ASTM Standards:*

D 3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Bacteriology[3]

D 5245 – 92 Practice for Cleaning Laboratory Glassware, Plasticware and Equipment used in Microbiological Analysis[3]

D 5465 – 93 Practice for Determining Microbial Counts From Waters Analyzed by Plating Methods[3]

E 691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method[4]

3. Summary of Guide

3.1 ASTM standard practices are referenced for use by producers and users to determine the potential utility of the nonconventional test. Users of tests who are unequipped for performing standard microbiological tests are given recommendations for seeking out microbiological laboratories that could perform collaborative studies to evaluate and verify the information generated with the nonconventional tests.

4. Significance and Use

4.1 This guide should be used by producers and potential producers of nonconventional tests to determine the accuracy, selectivity, specificity, and reproducibility of the tests, as defined in Practices E 691 and D 3870. Results of such studies should identify the limitations and indicate the utility or applicability of the nonconventional test, or both, for use on different types of samples.

4.2 Nonconventional test users and potential users should employ this guide to evaluate results of the nonconventional test as compared to their present methods. Practices D 5245 and D 5465 should be reviewed in regards to the conventional microbiological methods employed. If conventional methods have not been used for monitoring the systems, then guidelines are included for obtaining microbiological expertise.

4.3 Utilization of a nonconventional test may reduce the time required to determine the microbiological status of the system and enable an improvement in the overall operating efficiency. In many cases, the findings of a significantly high level of bacteria indicates the need for an addition of an

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

[3] *Annual Book of ASTM Standards,* Vol 11.02.

[4] *Annual Book of ASTM Standards,* Vol 14.02.

antimicrobial agent. By accurately determining this in a shorter time period than by conventional methods, treatment with antimicrobial agents may circumvent more serious problems than if the treatment were postponed until conventional results were available. If the antimicrobial treatment program relies on an inaccurate nonconventional test, then unnecessary loss of product and problems associated with inappropriate selection or improper dosing with antimicrobial agents would exist.

4.4 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.

5. Procedures

5.1 In order to determine the utility of the nonconventional test, evaluate and compare the results to those obtained with a previously accepted standard method. Often, the Heterotrophic Plate Count is entirely satisfactory for this purpose **(2)**; however, understand its limitations before it is used as the basis for evaluating methods that measure other parameters indicative of microbial life (metabolic activity, concentration of cell constituents, or whole cell numbers). The variety of methods used for the Heterotrophic Plate Count are listed in Table 1. When this method is not a suitable standard, use 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. 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 nonconventional method achieves the objectives defined in the scope of the procedure.

5.2 A knowledge of standard microbiological technique is required for this procedure. If that expertise is not currently available in-house, then consult an outside testing laboratory. Many industrial microbiology laboratories are certified for the analysis of drinking water by the EPA or the state government (a listing of these laboratories can be obtained from the regional EPA office or the state government). There are also other microbiology laboratories that specialize in processing samples from different industries; these are often listed as "Laboratories—Testing" in the telephone book. It is important that this document be referenced when undertaking an evaluation with an outside laboratory.

5.3 For each method, first make an enumeration of all major sources of variability. For example, if a nonconventional test method is involved and if more than a single analysis can be conducted with a single test, consider the variability within and between tests. For plates, it is important to consider the variability between plates obtained from aliquots of the same sample. It is also important to prepare samples covering the entire range of values (for example, counts per milliliter) of interest. Each such value is referred to as a level. Thus, the levels must cover the range of interest.

5.4 At each level, analyze replicate samples, both by the method under study, and by the standard method. The number of replicates depends on the number of sources of variability. Thus, in the previous-mentioned example of nonconventional test, it would be advisable to analyze at each level at least two replicates of each (preferably more) in at least two nonconventional tests (preferably more). At the same time, analyze replicates by the Heterotrophic Plate Count, resulting in several replicate plates. The scheme shown in Table 2 illustrates such a procedure; in this case, three replicates are analyzed at any given level using three nonconventional tests, while five replicate plates are counted by the Heterotrophic Plate Count. (These numbers will vary according to the method.)

5.5 Using the example of Table 2, the data of the new method would be analyzed and compared with the Heterotrophic Plate Count method for determining precision, as well as (*1*) within-test variability; (*2*) between-test variability; and (*3*) between-plate variability.

5.6 Again, using the example of Table 2, the nine values by the new method and the five values by the Heterotrophic Plate Count are averaged for all levels and then plotted. A curve, using appropriate statistical procedures, must then be fitted to these points. This curve is the calibration line of the new method versus the Heterotrophic Plate Count, and it can be

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

	Water 5	Dairy 6	Environment 7	Food 8	Cosmetic 8	Paper 9	Pharmaceutical 10
Media	TGE, SM, R2A or m-HPC	SM	SM or TGE	SM	ML	TGE	SCD
Dilution, H_2O	KH_2PO_4+ $MgCl_2$	KH_2PO_4	KH_2PO_4	KH_2PO_4	MLB	H_2O	KH_2PO_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 Letheen Agar
MLB = Modified Letheen 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)

TABLE 2 Scheme for Analysis at a Given Level

Test	New Method		Heterotrophic Plate Count	
	Replicate Within Test	Determination	Plate	Determination
1	1	1	1	1
	2	2	2	2
	3	3	3	3
			4	4
			5	5
2	1	4		
	2	5		
	3	6		
3	1	7		
	2	8		
	3	9		

used to convert values obtained by the new method into equivalent units of the Heterotrophic Plate Count.

6. Report

6.1 The standard deviations obtained by the new method can be converted, by appropriate statistical procedures, into equivalent units of the standard method by using the calibration line for conversion. A comparison with the standard method can then be made to determine the precision of the new method.

6.2 In view of the complexity of the problem and variety of situations that can arise, it is not possible to recommend further 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* **(3)** and in Chapter 14, "The Comparison of Method of Measurements," of *The Statistical Analysis of Experimental Data* **(4)**.

7. Precision and Bias

7.1 A precision and bias statement cannot be made for this guide.

REFERENCES

(**1**) Roszak, D. B., and Colwell, R. R., "Survival Strategies of Bacteria in the Natural Environment," *Microbiological Reviews*, Vol 51, No. 3, Sept. 1987, pp. 365–379.

(**2**) Buck, J. D., "The Plate Count in Aquatic Microbiology," *Symposium on Native Aquatic Bacteria: Enumeration, Activity, and Ecology*, edited by J. W. Costerton and R. R. Colwell, *ASTM STP 695*, ASTM, 1979, pp. 19–28.

(**3**) Youden, W. J., and Steiner, E. H., *Statistical Manual of the Association of Official Analytical Chemists*, Second Printing, Association of Official Analytical Chemists, Arlington, VA 22209, 1979.

(**4**) Mandel, J., *The Statistical Analysis of Experimental Data*, Dover, 1984.

(**5**) "Standard Methods for the Examination of Water and Wastewater," American Public Health Association, New York, NY, 19th ed., 1995 or most current.

(**6**) "Standard Methods for the Examination of Dairy Products," American Public Health Association, New York, NY, 16th ed., 1993 or most current.

(**7**) "Microbiological Methods for Monitoring the Environment," Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio, *EPA 600/8-78-017*, December 1978.

(**8**) FDA Bacteriological Analytical Manual, Food and Drug Administration Staff, 1995, AOAC International, Arlington, VA, 8th ed., or most current.

(**9**) "Microbiological Examination of Process Water and Slush Pulp," (proposed review of Official Method T631 om-79), Technical Association of the Pulp and Paper Industry, Technology Park, Atlanta, GA, April 5, 1984, or most current.

(**10**) "Microbial Limits-Total Aerobic Microbial Count," *U.S. Pharmacopoeia XXIII-National Formulary*, U.S. Pharmacopoeia Convention, Inc., Rockville, MD, 1995 or most current.