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Standard Guide for Interlaboratory Studies for Microbiological Test Methods[1]

This standard is issued under the fixed designation D7847; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

Microbiological parameters present a number of unique challenges relative to chemical and physical test methods apropos of the development of precision and bias terms. A number of these challenges are discussed in Guide E1326. As a working group (WG) we first grappled directly with some of these issues during the development of Practice D6974. The drafts balloted at the D02.14 subcommittee level in February and June 2002, were balloted with the document identified as a Method. Moreover, the proposed Method was drafted as a harmonized document with the Energy Institute's (EI) Method IP 385. When the item was balloted at D02 level, members of D02.94 compelled us to change the title from Method to Practice. The argument was that ASTM Methods list single series of steps that lead to a measurable result (a bit of data; quantitative, semi-quantitative or qualitative). Because D6974 provides for the selection of different sample volumes (based on the estimated culturable population density) and different growth media (based on the sub-population to be quantified), it would only be accepted as an ASTM Practice; not a Method. This issue of performing interlaboratory studies for culture methods will be discussed below.

Since Practice D6974 was approved, four microbiological test methods have been approved by ASTM: D7463, D7687, D7978, and D8070.

Because these methods measure the concentration of a biomarker molecule or microorganisms, the issues that are relevant to ILS are similar to, but somewhat different than those that affect ILS for culture methods. Beckers[2] investigated microbiological test method interlaboratory studies, but advised several measures that are either impractical for or not relevant to the methods that have been developed within D02: *(1)* Freeze inoculated samples after dispensing into portions for shipment to participating labs; *(2)* Use a single organisms challenge; *(3)* Add the challenge microbe to a sample matrix in which it is likely to proliferate.

This guide will list key issues that must be addressed when designing ILS for Methods intended to measure the microbial properties of fuels and fuel-associated waters.

1. Scope*

1.1 Microbiological test methods present challenges that are unique relative to chemical or physical parameters, because microbes proliferate, die off and continue to be metabolically active in samples after those samples have been drawn from their source.

1.1.1 Microbial activity depends on the presence of available water. Consequently, the detection and quantification of microbial contamination in fuels and lubricants is made more complicated by the general absence of available water from these fluids.

1.1.2 Detectability depends on the physiological state and taxonomic profile of microbes in samples. These two parameters are affected by various factors that are discussed in this guide, and contribute to microbial data variability.

1.2 This guide addresses the unique considerations that must be accounted for in the design and execution of interlaboratory studies intended to determine the precision of microbiological test methods designed to quantify microbial contamination in fuels, lubricants and similar low water-content (water activity <0.8) fluids.

[1] This test method is under the jurisdiction of ASTM Committee D02 on Petroleum Products, Liquid Fuels, and Lubricants and is the direct responsibility of Subcommittee D02.14 on Stability, Cleanliness and Compatibility of Liquid Fuels.

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[2] Beckers, H. J., "Precision Testing of Standardized Microbiological Methods," *Journal of Testing and Evaluation*, JTEVA, Vol. 14, No. 6, November 1986, pp. 318–320.

***A Summary of Changes section appears at the end of this standard**

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D156 Test Method for Saybolt Color of Petroleum Products (Saybolt Chromometer Method)
D1129 Terminology Relating to Water
D4012 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water
D4175 Terminology Relating to Petroleum Products, Liquid Fuels, and Lubricants
D6300 Practice for Determination of Precision and Bias Data for Use in Test Methods for Petroleum Products, Liquid Fuels, and Lubricants
D6469 Guide for Microbial Contamination in Fuels and Fuel Systems
D6974 Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures
D7463 Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Fuel, Fuel/Water Mixtures, and Fuel Associated Water
D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing
D7687 Test Method for Measurement of Cellular Adenosine Triphosphate in Fuel and Fuel-associated Water With Sample Concentration by Filtration
D7978 Test Method for Determination of the Viable Aerobic Microbial Content of Fuels and Associated Water—Thixotropic Gel Culture Method
D8070 Test Method for Screening of Fuels and Fuel Associated Aqueous Specimens for Microbial Contamination by Lateral Flow Immunoassay
E1259 Practice for Evaluation of Antimicrobials in Liquid Fuels Boiling Below 390 °C
E1326 Guide for Evaluating Non-culture Microbiological Tests
E1601 Practice for Conducting an Interlaboratory Study to Evaluate the Performance of an Analytical Method
E2756 Terminology Relating to Antimicrobial and Antiviral Agents

[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.

2.2 *Energy Institute Standard:*[4]

IP 385 Viable aerobic microbial content of fuels and fuel components boiling below 390 °C—Filtration and culture method

3. Terminology

3.1 For definition of terms used in this guide refer to Terminologies D1129, D4175 and E2756, and Guide D6469.

3.2 *Definitions:*

3.2.1 *free water, n*—water in excess of that soluble in the sample and appearing in the sample as a haze or cloudiness, as droplets, or as a separated phase or layer. **D156**

3.2.2 *specific concentration, n*—the fraction of a cell constituent as determined on a per cell basis.

3.2.2.1 *Discussion*—The specific concentration can be expressed as weight to weight, weight to volume or volume to volume basis. Enzymes are commonly reported in terms of their activity relative to a reference standard.

3.3 *Acronyms:*

3.3.1 *ATP*—adenosine triphosphate

3.3.2 *DNA*—deoxyribonucleic acid

3.3.3 *ILS*—interlaboratory study

3.3.4 *RNA*—ribonucleic acid

4. Determining Precision and Bias

4.1 *Bias Testing:*

4.1.1 There are no generally accepted reference standards for microbial cell constituents or for culture enumeration by viability test methods.

4.1.2 Consequently, bias cannot be determined for non-culture methods.

4.1.3 Data obtained from testing an accepted non-culture parameter or culture method can be compared against data obtained using a proposed new method.

4.1.3.1 Such comparisons are useful for benchmarking newly measure parameters against historically measure ones.

4.1.3.2 Because bioburden is not a condition of state and because individual microbial parameters respond to sources of variation differently, comparison of a new method's test results against those of a preexisting method cannot be used to determine the bias of either method.

4.2 *Precision Testing:*

4.2.1 *Repeatability Testing:*

4.2.1.1 *Sample Heterogeneity:*

(1) Unlike chemical and physical characteristics which are generally uniform throughout a well-mixed sample, microbes are discrete bodies that are dispersed in the medium.

(2) In contrast to inanimate particles, microbes typically form aggregates in which individual cells are bound to one another within a polymeric matrix that is difficult to remove without also damaging cells.

(3) Microbes are similar to inanimate particles in that their settling rate within a medium follows Stoke's law.

[4] Available from Energy Institute, 61 New Cavendish St., London, WIG 7AR, U.K., http://www.energyinst.org.uk.

(4) Heterogeneous distribution of microbes within a medium is likely to be a significant source of variability relative to other factors affecting test method repeatability.[5]

(5) Microbes require free-water in order to be metabolically active (see 1.2).

(a) In a given fuel system, microbial population densities tend to be greatest at interfaces; particularly the fuel-water and fuel-system-surface interfaces.

(b) Population densities within these interface zones are also heterogeneous.

(c) In order to minimize variability due to sample heterogeneity, replicate samples should be recovered from as close to the same locus as possible.

4.2.1.2 *Microbial Population's Physiological State:*

(1) The physiological state of a challenge population is largely dictated by physicochemical conditions, population lifecycle stage in closed systems, flow and shear in open and semi-open systems, and the similarities between the challenged microcosm and source microcosm.

(2) The specific concentration of many microbial cell constituents varies in response to the physiological state of a challenge population.

(3) Factors affecting the physiological state of a population also tend to affect the population's culturability.

(4) Guidance provided in Practices D6300 and E1601 minimize the impact of physiological state on repeatability statistics.

4.2.2 *Intermediate Precision Testing:*

4.2.2.1 Microbiological parameters are very perishable.

(1) Practice D7464 provides guidance on the maximum acceptable delays between sample collection and test initiation. However, individual methods can specify acceptable conditions and delays between sampling and the initiation of analysis.

(2) The history of a sample between time of collection and test initiation can affect population densities and physiological state substantially.

(3) Differences in sample histories (4.2.2.1*(2)*) can contribute to variability that eclipses variability due to differences in instrumentation, analytical technique or both.

(4) Factors affecting the state of microbial populations in samples include, but are not limited to: temperature, oxygen availability, chemical composition of sample medium, composition of sample container, degree of ullage space.

4.2.2.2 In order to minimize the potential contribution of disparate sample histories to reproducibility variability, it is advisable to conduct ILS either at a single location or at several closely located facilities.

4.2.2.3 The ILS design should include detailed instructions designed to minimize differences in sample histories between the time that participant subsamples are prepared and testing is initiated.

4.2.2.4 When all testing is performed at a single facility, operator/apparatus repeatability can be determined. Although the statistical computations are the same as those prescribed in Practice D6300, test plan for a single site study does not satisfy all of the reproducibility conditions stipulated in D6300, test result variability between operators and apparatus setups is not the same as reproducibility. Consequently, the test method's variability is reported as operator/apparatus repeatability.

[5] Passman, F. J., English, E., Lindhardt, C., "Using Adenosine Triphosphate Concentration as a Measure of Fuel Treatment Microbicide Performance," Morris, R. E., Ed., Proceedings of the 10th International Conference on the Stability and Handling of Liquid Fuels, Oct. 7-11, 2007, Tucson, AZ. Available at www.iash.net.

5. Culture Methods

5.1 *Selecting Test Organisms:*

5.1.1 *Microbial Diversity:*

5.1.1.1 The number of different types of microbes recovered from microbially contaminated fuel and fuel-associated waters is known to range from single to dozens of different taxa.

5.1.1.2 Any given nutrient medium and set of growth conditions will select for a sub-population of the total microbial population (5.2.1).

5.1.1.3 Non-culture methods have identified the presence of microbial contaminants that have yet to be cultivated on growth media.

5.1.1.4 Depending on the method's scope, the appropriate options for precision testing include:

(1) Single culture from type culture collection—most appropriate when the method is designed to detect a specific microbial taxon.

(2) Mixed population of type collection cultures—provides a basis for evaluating the recovery of microbes representing a more diverse population (Practice E1259).

(3) Uncharacterized population obtained from one or more contaminated systems—most closely reflects field conditions.

(4) Commercially available uncharacterized mixed population of microbes known to metabolize fuel components (for example: fats, oils and greases).

(5) A commercially available population of microbes that are capable of producing a reliable signal detectable by the instrument detector and will survive at least for 24 h in fuel (hydrocarbon) environment.

(6) Field samples.

(7) Combinations of two or more of the above.

NOTE 1—No collection of contaminated fuels or fuels and fuel-associated waters is likely to be truly representative of microbial diversity in fuel systems.

5.1.2 *Physiological State (4.2.1.2):*

5.1.2.1 When a challenge population is transferred from the source medium to the test sample, it is likely that the population will need to acclimate to its new physicochemical environment.

5.1.2.2 This acclimation period can be reduced—but not totally eliminated—by ensuring that challenge populations are pre-acclimated to conditions by preculturing them in microcosms that are as similar as possible to the conditions of the sample that will be used for precision testing.

NOTE 2—During the acclimatization period microbes are likely to regain full metabolic activity in zones in which free-water is present (4.2.1.1*(5)*). If there is no free-water in the sample, microbes are likely to become metabolically dormant.

5.1.3 *Generation Time:*

5.1.3.1 Commonly, microbes with generation times ≤1 h are used for culture tests so that colonies are visible within 24 h to 48 h.

NOTE 3—The generation times of different microbes in uncharacterized populations is neither known nor uniform among microbes. Generation times vary among types of microbes and environmental conditions.

5.2 *Selecting Culture Media:*

5.2.1 Given the physiological diversity of Eubacteria, Archeae, and Fungi, no single nutrient medium formulation or set of incubation conditions will support the proliferation of all cells in a challenge population.

5.2.2 Consequently, a negative bias is assumed for all culture test methods.

5.2.2.1 It is generally accepted that only a small fraction of microbial taxa have been cultured.

5.2.2.2 There are no reference standards against which to quantify a culture method's bias (Guide E1326), consequently, only precision statistics can be developed for culture methods.

5.2.3 Culture media selection is typically defined within a microbiological test method to ensure that the test results are consistent with the method's objectives (IP 385 and Practice D6974).

5.3 *Separating Microbes from Sample:*

5.3.1 Sample carryover can interfere with culturability.

5.3.1.1 Nutrients carried over with the sample can enable microbes that might not otherwise elaborate into colonies to proliferate on the chosen culture medium.

5.3.1.2 Inhibitory chemicals (including, but not limited to microbicides) can prevent viable microbes from elaborating into colonies.

5.3.2 Method and practice protocols should include provisions to minimize interferences due to the presence of sample fluid.

6. Non-Culture Methods

6.1 *Common Issues Shared with Culture Methods:*

6.1.1 The factors discussed in 5.1.1, 5.1.2 and 5.3 also apply to non-culture methods.

6.2 *Issues Unique to Non-culture Methods:*

6.2.1 In particular, the specific concentration of individual constituents (for example ATP – Methods D4012, D7463, D7687, and D8070) will vary with the organisms' physiological state (4.2.1.2*(2)*).

6.2.2 Additionally, genetic constituents (DNA and RNA) vary qualitatively as well as quantitatively based on the taxonomic make up (diversity) of the microbial population in the sample.

7. Sample Types

7.1 *Microbes in Fuels:*

7.1.1 As discussed in 4.2.1.1*(5)*, microbes concentrate where there is free-water.

7.1.2 Microbes recovered from fuel-phase samples are likely to be dormant.

7.1.3 During culture testing (IP 385 and Practice D6974) organisms are placed in a nutrient rich, available free-water environment, which permits cells to transform from dormancy into a metabolically active (vegetative) state.

7.1.4 During non-culture testing, cells normally do not have the opportunity to undergo the transformation described in 7.1.3. Consequently the specific concentrations of many cell constituents are typically less than they are in vegetative cells.

7.1.5 The phenomena listed in 7.1.1 through 7.1.4 explain why fuel specifications do not include microbiological quality control criteria.

7.1.5.1 The probability of recovering microbes in fuel-phase samples is small relative to the probability of recovering microbes from fuel-associated waters.

7.1.5.2 Specifications are for fuels; not fuel-associated water; although uncontrolled microbial contamination in fuel-associated water can contribute to fuel and fuel system deterioration (Guide D6469).

7.2 *ILS Options:*

7.2.1 *Water-free Fuel Samples:*

7.2.1.1 Suspending challenge microbes into water-free fuel is likely to cause substantial negative bias to both culture and non-culture tests. Osmotic shock can cause cell lysis, and induce non-lysed cells to transition into a dormant state (7.1).

7.2.1.2 Microbiology is primarily a science of population dynamics. The impact of suspending challenge microbes into water-free fuel is to increase sample heterogeneity.

7.2.2 *Fuel-over-water Microcosms:*

7.2.2.1 Per 4.2.1.1*(5)*, microbes in fuel systems proliferate where free-water is available.

7.2.2.2 Fuel-phase samples in two-phase systems will have a much lower biomass concentration than will aqueous-phase samples.

7.2.2.3 Recognizing that the aqueous phase is zone of control for microbial contamination in fuel system, Practice E1259 prescribes quantifying microbial loads only in the aqueous-phase on two-phase microcosms. A similar argument could be made for fuel microbiology test methods.

8. Options

8.1 *Develop Repeatability Data Only:*

8.1.1 *Form and Style for ASTM Standards* § A21.5.4 provides for methods for which either repeatability or reproducibility cannot be determined:

"If it is not possible to provide a statement on precision (repeatability or reproducibility) as directed in A21.2, use a statement such as the following:

"Precision—It is not possible to specify the precision of the procedure in Test Method X0000 for measuring (insert here the name of the property) because (insert here the reason or reasons)."

8.1.2 Given the combination of factors that prevent the preparation and dissemination of samples that will be nearly identical when analyzed, it might be appropriate to invoke A21.5.4 for all fuel microbiology test methods.

8.2 *Developing Single Operator Repeatability and Operator/Apparatus Repeatability Data:*

8.2.1 The factors discussed in 4.2.2 make it clear that the preparation of samples at one facility for shipment to participating laboratories is an inadequate process for microbiology method interlaboratory study.

8.2.1.1 When an ILS is performed at a single facility, the repeatability statistic shall be reported as *Repeatability*: The difference between repetitive results obtained by the same operator in a given laboratory applying the same test method with the same apparatus under constant operating conditions on identical test material within short intervals of time would in the long run, in the normal and correct operation of the test method, exceed the intermediate repeatability coefficient only in one case in 20.

NOTE 4—Practice D6300 stipulates that replicate specimens be drawn from individual sample containers. Actual bioburden differences among replicate samples can eclipse variability due to the test protocol. Consequently, replicate specimens should be drawn from a single sample container (see 8.2.2.2).

8.2.1.2 When an ILS is performed at a single facility, the reproducibility statistic is normally reported as *Intermediate Precision—Between Operator/Apparatus Repeatability*: The difference between two single and independent results obtained by different operators applying the same test method in same laboratory using different apparatus on identical test material within short intervals of time would, in the long run, in the normal and correct operation of the test method, exceed intermediate repeatability coefficient only in one case in 20.

8.2.2 Presuming that primary sample (sample from which participant sub-samples are drawn) heterogeneity issues can be addressed, is by conducting the ILS at a single facility or a limited number of facilities that are located sufficiently close to ensure that the time delay and variability of conditions to which sub-samples are exposed between sub-sample preparation and test initiation are minimized.

8.2.2.1 This approach can mean that multiple ILS participants share reagents and instruments (as long as the methods are the same. Different methods may require different skill sets and minimum amount of hands-on time to ensure a minimum level of proficiency so as to not bias the ILS.)

8.2.2.2 The prescription in Practice D6300 (6.5.3.4), requiring that replicate subsamples be tested in a blind fashion (that is, each test specimen be drawn from a separate, random-digit identified container to ensure that the operator does not know that replicate tests are, in fact, replicates) is untenable for the performance of microbiology tests.

(a) Once samples are divided among different containers, the population dynamics within each container is likely to differ; causing substantial bioburden variability among replicate containers.

(b) Based on the substantial contribution of bioburden variability to the observed variability among putatively replicate subsamples, the methodrelated variability is likey to be eclipsed by bioburden variability.

(c) The phenomena described in 8.2.2.2(a) and (b) are illustrated by the data from ILS 1259 (Test Method D7687) and ILS 1260 (Test Method D8070). Details are provided in Appendix X1. For both test methods, preliminary repeatability precision was substantially greater than the intermediate precision—repeatability. In both cases, during the preliminary ILS, replicate specimens were taken from a single container, but during the full ILS, replicate specimens were taken different containers, in accordance with D6300 (6.5.3.4). Both ILS were performed on the same sample set. When the individual sample data (n = 192) from the two parameters were compared, the test results agreed for 83 % of the samples. This degree of agreement between two different parameters supports the hypothesis that bioburden variability among replicate containers eclipsed the variability attributable solely to the methods' protocols.

(d) For microbiological test method ILS, replicate specimens shall be drawn from a single sample container.

8.2.2.3 Coordination with Subcommittee D02.94 will be needed to ensure that 4.2.2.1 accommodations do not adversely affect the acceptability of the ILS data set.

8.2.3 Microbiology test methods are nominally fuel-grade independent. D02.94 guidance should be sought to determine the number of different fuel and blend- stock grades that must be included for a valid ILS.

9. Keywords

9.1 bioburden; biodeterioration; biodiesel; biofuels; biomass; diesel; fuel; fuel-oil; fungi; gasoline; interlaboratory studies (ILS); microbial contamination; microbiology; microorganisms

APPENDIX

(Nonmandatory Information)

X1. ASSESSMENT OF SOURCES OF VARIABILITY: A CASE STUDY

X1.1 As explained in 6.1, 7.2, and 8.2, relevant sources of variation other than experimental error can contribute substantially to microbiological test method repeatability and reproducibility variability.

X1.2 Preliminary and final ILS repeatability statistics from Test Methods D7687 and D8070 illustrate the issues:

X1.2.1 *Test Method D7687:*

X1.2.1.1 Preliminary intermediate precision—repeatability (r): in fuel and fuel associated water:

$$r[\text{cATP}](\text{in pg/ mL}) = 0.33X \qquad (X1.1)$$

where:

X = average of triplicate tests

The sample types and raw data are provided in Table X1.1 and Table X1.2, respectively.