Method Validation of U.S. Environmental Protection Agency Microbiological Methods of Analysis

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Foreword

The U.S. EPA Science Policy Council (SPC) established the Forum on Environmental Measurements (FEM) in April 2003. The FEM is a standing committee of senior EPA managers who provide EPA and the public with a focus for addressing measurement and methods issues with multiprogram impact. Action teams are commissioned by the FEM to address specific issues. The Method Validation Team was formed in October 2003 and tasked with developing Agency-wide, internal guidance for validating and peer reviewing EPA methods prior to publication for general use. This document contains guidance for microbiological methods of analysis.

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TABLE OF CONTENTS

Page No.

1.0 INTRODUCTION 1-1

1.1 Microbiology at EPA 1-1

1.2 Purpose 1-2

1.3 Intended Audience 1-2

1.4 Scope of the Guidance 1-2

2.0 GENERAL GUIDELINES FOR METHOD VALIDATION 2-1

2.1 Introduction 2-1

2.2 Method Selection 2-1

2.3 Method Optimization 2-2

2.4 Development of Operational Limits and Within-Laboratory Method Performance Attributes 2-3

2.4.1 Specificity and Sensitivity 2-4

2.4.2 Precision 2-5

2.4.3 Accuracy and Bias 2-6

2.4.4 Limit of Detection (LOD) 2-7

2.4.5 Linearity 2-7

2.4.6 Calibration and Other Performance Attributes of Absolute Quantitative Test Methods 2-8

2.4.7 Special Considerations for Culture-based Microbiological Test Methods 2-9

2.4.8 Other Operational Limits and Quality Control Measures 2-9

2.5 Writing the Method 2-10

2.6 Multi-laboratory Validation Studies 2-11

2.6.1 Tier 1 Validation 2-11

2.6.2 Tier 2 Validation 2-11

2.6.3 Tier 3 Validation 2-11

2.6.4 Types of Multi-laboratory Validation Studies 2-12

2.6.5 Participating Laboratories 2-13

2.6.6 Test Materials 2-15
2.6.7 Replication of Test Materials 2-16
2.7 Development of a Study Plan and Study Implementation 2-17
2.8 Reporting Results 2-17
3.0 REFERENCES 3-1

Appendix A – EMMC Format for Writing a Method
1.0 Scope and Application
2.0 Summary of Method
3.0 Definitions of Method
4.0 Interferences
5.0 Safety
6.0 Equipment and Supplies
7.0 Reagents and Standards
8.0 Sample Collection, Preservation and Storage
9.0 Quality Control
10.0 Calibration and Standardization
11.0 Procedures
12.0 Data Analysis and Calculations
13.0 Method Performance
14.0 Pollution Prevention
15.0 Waste Management
16.0 References
17.0 Tables, Diagrams, Flowcharts, and Validation Data

Appendix B – Interlaboratory Collaborative Study
Validation Protocol for the Quantitative Three Step Method

Appendix C – Guidelines for the Development and Validation of Nucleic Acid Amplification (PCR) based Microbiological Method
Introduction
1.0 The PCR Method: General Background
2.0 Method Selection
3.0 Method Development and Optimization
3.1 Design of Primers and Probes
3.2 Optimization of Reaction Conditions
3.3 Sample Preparation
3.4 PCR Inhibition Control
3.5 Sample Processing Control
3.6 Preparation and Use of Standards for Quantitative Real-time PCR Applications
3.7 Real-time Quantitative PCR Data Analysis
4.0 Quality Assurance and Quality Control (QA/QC)
5.0 PCR Method Performance Criteria
5.1 Specificity and Sensitivity
5.2 Precision
5.3 Accuracy and Bias
5.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)
5.5 Sample Limit of Detection (SLOD)
5.6 Linearity, Range of Quantification (ROQ) and Amplification Efficiency
5.7 Ruggedness

6.0 Multilaboratory Validation Studies
6.1 Influence of PCR Platform and Reagent System
7.0 Potential Differences between PCR Chemistries
8.0 References

Appendix D – Guidelines for the Validation of Efficacy Test Methods
Introduction
1.0 Selection of Test Method to Generate Product Efficacy Data
2.0 Validation of Efficacy Test Methods and Study Plan Preparation
3.0 Pre-Validation Considerations: Factors Affecting Efficacy of Antimicrobial Products
4.0 Desirable Attributes of a Validated Test Method
5.0 Method Performance Characteristics
6.0 References

Appendix E – The Method Approval Process
Method Approval through the Rule Making Process
1.0 Alternate Method Approval Processes
1.1 Alternate Test Procedure (ATP) Program
2.0 Expedited Methods Approval
3.0 Reference Methods and Other Approved Methods
4.0 References

List of Figures

Figure 1-1: Overall Method Validation and Approval Process 1-4
Figure 2-1: Margin of Error for a Method with 80 % Sensitivity Rate and Confidence Interval of 90% 2-14

1.0 INTRODUCTION

1.1 Microbiology at EPA

Microorganisms are ubiquitous in the environment. As such, the study of these organisms and their impact on human health through environmental exposures is an important part of EPA's mission. Consequently, the Agency is extensively involved in the study and monitoring of microorganisms in a variety of matrices, including air, water, soil, sludge and surfaces (fomites). In addition, to better manage pathogenic organisms in the environment, EPA is heavily invested in determining the impact of various treatment and decontamination procedures on microorganisms. The data generated by these efforts help inform EPA in the development of regulations and improved pathogen management approaches.

With specific regard to drinking and source waters, the Agency develops regulations designed to address the issue of microbial contamination. The agency develops methods that are then validated and used to support regulations. In addition, the agency also develops consensus methods, which are not used by industry for compliance but to provide advisory information/data. Consensus methods are often developed through collaboration between industry and EPA to assist the industry in monitoring un-regulated parameters. A good example of the Consensus Method process is the Microscopic Particulate Analysis (MPA) (EPA 910/9-92-029, 1992), a microscopic technique developed to assist water utilities and State regulatory agencies in determining if groundwater sources are under the direct influence of surface water. For both implementation of regulations and advisory purposes, EPA publishes microbiological methods used by public water utilities, academia, industries and other government agencies. Regardless of the purpose, reliable, and accurate methods are needed to ensure the validity of the data collected. Methods used for these purposes therefore must be validated before they are published as EPA methods.

Method validation is defined as a process that demonstrates the suitability of an analytic method for its intended purpose (Green 1996). This document is intended to provide general guidance for the validation of microbiological methods likely to be used in future EPA methods. This document is designed to be applicable to all methods pertaining to assaying environmental microorganisms as well as to efficacy testing of antimicrobial agents. In exploring requirements to validate a specific method, the EPA Forum on Environmental Measurements (FEM) Microbiology Action Team concluded that the provision of specific guidance on how to conduct

validation studies for every method of analysis was beyond the scope of the document. Therefore, detailed validation protocols applicable to specific methods are not covered. Instead, guidelines for general validation are provided with some emphasis on certain technologies. Wherever appropriate, validation protocols developed by organizations and used by EPA are referenced; e.g. International Standards Organization (ISO), American Society for Testing and Materials (ASTM) International, and Association of Analytical Chemists (AOAC) International.

As new microbiological methods of analysis are developed, this document may be revised. This text, therefore, should be treated as a living document.

1.2 Purpose

The purpose of this document is to provide agency-wide guidance on validating microbial methods, thus promoting consistency in the area of measurement methodology and policy across EPA program offices and regions.

1.3 Intended Audience

This guidance is intended for internal use by EPA personnel who are responsible for the development, implementation and review of microbiological methods of analysis used in environmental testing. These methods may be published as serially numbered EPA methods, or incorporated by reference in regulations or could be used purely for research efforts by EPA's Office of Research and Development and the EPA Regions. It is recognized that this document may also be used by clients, contractors or other interested parties who, upon reviewing an EPA method for potential use, are interested in the Agency's process for validation, approval, and acceptance of EPA methods.

1.4 Scope of the Guidance

This guidance is intended for new EPA microbiological methods that have not yet undergone validation prior to being published as EPA methods or adapted as Agency-accepted regulatory standards. This guidance includes validation principles for a range of analytes such as whole microbes or nucleic acids. A typical method involves sample collection, sample processing, extraction or isolation (microbes or nucleic acid) procedures, and analytical detection

of analytes. Validation issues with extraction procedures and detection are addressed in this guidance. A separate guidance document is being prepared for microbiological sampling.

The primary focus of this document is validation guidelines for microbiological methods. Emphasis is on issues associated with PCR methods and efficacy test methods for antimicrobials in Appendices C and D, respectively. The guidance also provides readers with an understanding of EPA's method approval process (Appendix E) after the methods are validated. The general flow depicting overall method validation and approval process is shown in Figure 1.

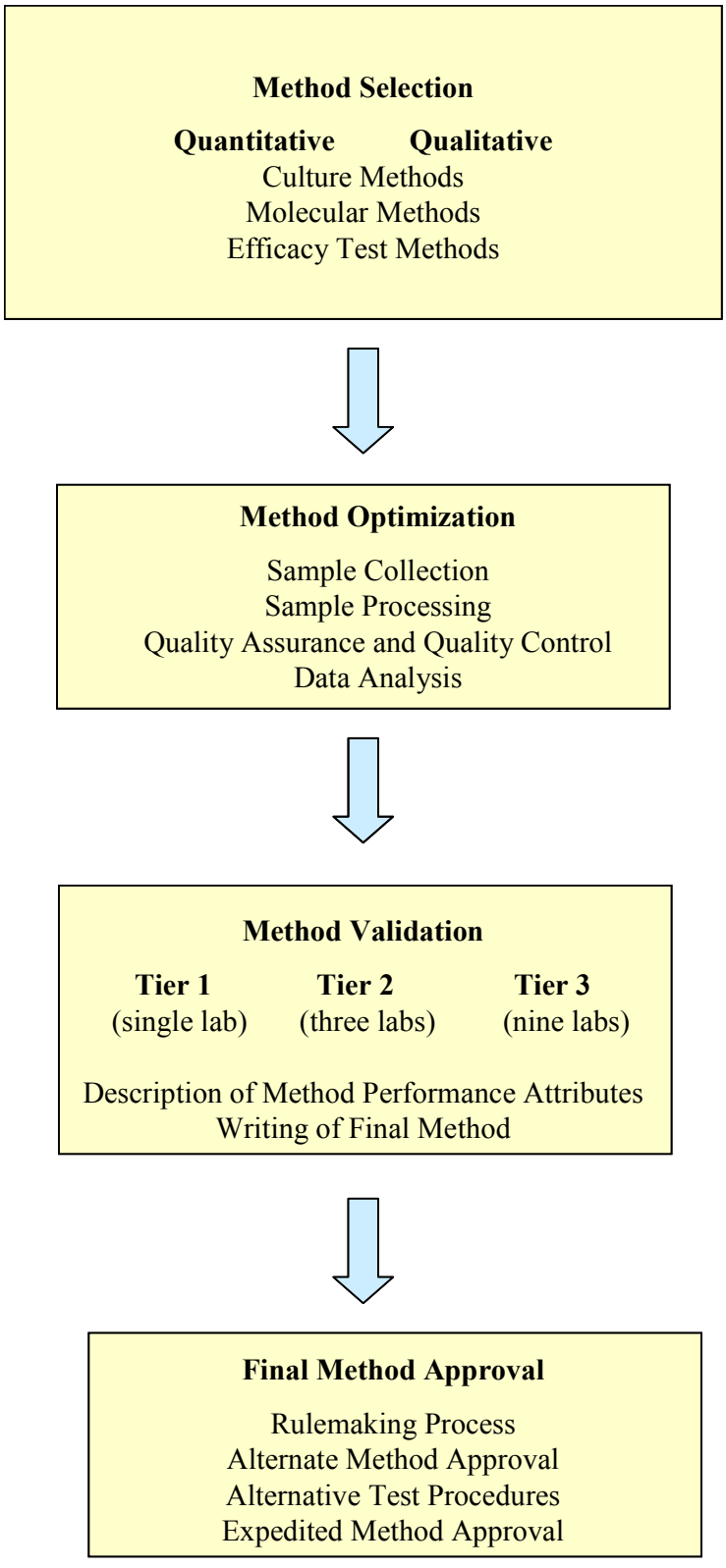

Figure 1. Overall Method Validation and Approval Process

2.0 GENERAL GUIDELINES FOR METHOD VALIDATION

2.1 Introduction

Method validation is the process of determining if a method is suitable for its intended purpose. Validation can be classified as primary validation and secondary validation according to its purpose. Primary validation is an exploratory process for establishing the operational limits and performance characteristics of a new, modified or otherwise inadequately characterized method. Secondary validation on the other hand, is the process of gathering evidence that a laboratory can meet the specifications established in primary validation (ISO 2000).

This section outlines a series of steps and procedures that are recognized as being integral to the selection and validation of an analytical method for an intended use. These steps are normally performed sequentially and procedural details may vary for different types of methods.

It should be noted that successful validation of any analytical method is not possible without thorough and systematic planning and preparation. A written study plan should be prepared for each step of the validation process and subjected to appropriate review prior to implementation. In addition, it is expected that all laboratories involved in each step of a validation process will have a Quality System or quality assurance (QA) program in place to ensure standardization of laboratory operations, as well as adequate quality control (QC) activities.

2.2 Method Selection

Some methods may not require formal validation. The decision on whether to proceed with validation will often be predicated on an expectation that a method will be widely implemented and/or be needed to support a regulatory requirement. It will also require a judgment about the expected capability of the method(s) under consideration to meet the requirements of its intended use. Information may already be available for the method(s) that can be used in the decision process. Other criteria to consider before pursuing validation include whether a method:

X Is based on sound underlying scientific principles

X Is applicable for routine analysis of samples

X Can detect analytes in the concentration range of interest

X Has sufficient specificity and sensitivity for intended use

X Can meet specific method performance criteria

X Has adequate QA/QC controls

X Can be performed with readily-available equipment

X Can be conducted for a reasonable cost

X Addresses the level of expertise required (e.g., are there technique-sensitive areas? Is specialized training required?)

X Contains necessary aspects of quality assurance (e.g., calibrated equipment, media quality, incubation conditions)

X Addresses biosafety concerns (e.g., are there specific biosafety practices necessary to handle the test pathogens?) (Fleming and Hunt, 2000; Centers for Disease Control, 2007).

2.3 Method Optimization

New or, in many instances, even established methods identified in the selection process may require optimization prior to their validation. Optimization involves the identification of specific factors employed in the method such as procedures, environmental conditions, and reagents most likely to introduce variability in results and the subsequent iterative testing of modifications to the method that minimize this variability. Terms commonly used to characterize the stability of a method's results in the presence of variable factors are ruggedness and robustness. Ruggedness and robustness are defined as the ability of a method to provide acceptable results in the presence of variable factors that are unavoidable and avoidable, respectively. Examples of generally unavoidable sources of variability can include different analysts, lots or preparations of reagents, and calibration of instruments. Examples of potentially avoidable sources of variability could include the use of reagents and/or instruments from different sources. In some cases, information about these differences may be desirable, since it can allow for more flexibility in the use of the methods.

For effective optimization, it is important to consider the whole method, both the sample collection and processing component, as well as the detection assay. A highly sensitive assay such as PCR is meaningless if the overall recovery of an organism is poor and/or assay inhibitors are not removed. The impact of the sample collection procedure on a microorganism's viability is also important to consider, depending on whether the goal of the method is to specifically detect viable organisms or not. Furthermore, while it is possible to optimize individual components of methods and thereby estimate a given method parameter such as sensitivity, it is critical to empirically determine this value using the complete method. Finally, methods should be optimized to minimize the variability in results caused by interferences from different sample matrices. When matrix effects are a cause of significant variability and/or when quantification of the analyte is sought, use of an internal control should be considered part of the method.

While a substantial amount of effort can be invested in the optimization process, it is beneficial, in minimizing problems that may be encountered in the subsequent steps of method validation, particularly in the final interlaboratory testing stage. Standardized experimental and statistical designs for testing the ruggedness and robustness of analytical methods are also available to facilitate these efforts (Youden and Steiner, 1975; Dols and Ambrecht, 1976).

2.4 Development of Operational Limits and Within-Laboratory Method Performance Attributes

Once an analytical method has been developed and optimized, the first step in validating it is to determine its operational limits and within-laboratory performance attributes within these limits that are relevant to the intended use. As with optimization, this process is often carried out in the laboratory where the method was developed, but it may also be conducted by the organization intending to implement the method. This process, often referred to as primary validation, should provide preliminary baseline specifications (numerical and descriptive) of the method's performance within the laboratory performing the tests. The performance attributes requiring determination may differ depending on the nature and applications of the method (e.g., culture- vs. microscopy-based, molecular- or chemistry-based, and qualitative vs. quantitative) as are the experimental designs that are best suited to make these determinations. A guideline for determining performance attributes of several different broad categories of method types are available (ISO 1994b, ISO 1994c, ISO 2000, US FDA 2001). To obtain examples of relevant

experimental designs that might be employed, new method developers should also consult the scientific literature for descriptions of similar previously validated methods. In many instances, different terms and definitions have been applied to the same performance attributes in the context of different types and applications of methods. With these caveats, some performance attributes and operational limits that require determination for primary validation of most analytical methods are provided below.

2.4.1 Specificity and Sensitivity

Sensitivity and specificity are terms that can have different definitions for different types or categories of analytical methods. In a general sense, we define these terms by the extent to which a method responds uniquely to the specified target organism or group of organisms. Specificity is the ability to discriminate between the target organism and other organisms. It is mathematically expressed as:

$$\text{Specificity} = TNC / (TNC + TPI) \qquad \text{Equation (1)}$$

Where:

TNC = Number of samples tested negative correctly
TPI = Number of samples tested positive incorrectly

Specificity for microbiology methods and media is traditionally demonstrated through the use of pure positive and negative control cultures. For example, appropriate American Type Culture Collection (ATCC) strains for several groups of enteric control culture bacteria are given in Section 5.1.6.4 of EPA's Manual for the Certification of Laboratories Analyzing Drinking Water, 5th Edition (U.S. EPA 2005). Positive cultures listed for Enterococci include *Enterococcus faecalis* ATCC 11700 and *Enterococcus faecium* ATCC 6057. Appropriate negative controls include *Staphylococcus aureus* ATCC 6538, *Esherichia coli* ATCC 8739 or 25922, and *Serratia marsecens* ATCC 14756. The definition of appropriate target and non-target control cultures or other standards for use in both validation and routine QC would be expected in the development of any new microbial method. In a robust method, a single target organism should be discernable in complex matrices containing potentially millions of non-target organisms.

Sensitivity is the proportion of target organisms that can be detected. It is mathematically expressed as:

$$\text{Sensitivity} = TPC / (TPC + TNI) \qquad \text{Equation (2)}$$

Where:

TPC = Number of samples tested positive correctly
TNI = Number of samples tested negative incorrectly (Deep 2006)

Data to calculate sensitivity are typically generated by repeated testing of serial dilutions of a "known" spike standard.

2.4.2 Precision

Precision can be defined as the closeness in agreement between independent test results obtained under stipulated conditions (ISO 1994a). This term is usually expressed as the variance, standard deviation, or coefficient of variation of a series of test results. Precision is often expressed as the percent coefficient of variation (%CV), where:

$$\%CV = (\text{standard deviation of measurements} / \text{mean}) \times 100 \qquad \text{Equation (3)}$$

The expected precision of culture-based microbial methods is typically derived mathematically based on the assumption that bacteria are distributed randomly in a well-mixed sample and follow a Poisson distribution. For methods involving direct counts, such as membrane filtration or plate counts, the 95% confidence limits around any result are given in Standard Methods (20th Ed.), 9222B.6.c. For both methods, quantitation is obtained by Most Probable Number (MPN) analysis of presence/absence tests of samples divided into multiple tubes or wells. Ninety-five (95) % confidence limits for results obtained by MPN tests are given in SM 9221C.2. Any new proposed culture-based method would report expected levels of precision.

Precision may be considered at four levels: within-lab repeatability, within-lab reproducibility, between-lab repeatability, and between-lab reproducibility. The first two levels

should be addressed in the primary validation of a method over the entire concentration range of the analyte that is expected to be relevant to its intended use.

Repeatability. Repeatability can be defined as the closeness in agreement between results of successive measurements of the same analyte carried out under the same conditions of measurement over a short interval of time. Repeatability is also termed intra-assay precision.

Suppose the within laboratory precision is S_r and the between laboratory precision is S_L. Then the precision S_R (including within and between) among laboratories is expressed as

$$S_R = \sqrt{S_r^2 + S_L^2} \qquad \text{Equation (4)}$$

Reproducibility. Reproducibility can be defined as the closeness of the agreement between the results of measurements of the same analyte carried out under variable conditions of measurement. For determination of within-lab reproducibility, some of the variable conditions that should be considered include different time intervals between analyses, analysts, lots or preparations of reagents, instruments, and different water matrices.

2.4.3 Accuracy and Bias

Accuracy can be defined as the closeness of the agreement between a test result and the accepted reference value, while bias can be defined as the difference between the expectation of the test results and a known or accepted reference value. The term "accuracy," when applied to a set of test results, has been more comprehensively defined as a combination of random components (related to random error) and a common bias component (related to total systematic error) associated with the method (ISO 1994a). As with precision, this bias component should be first characterized at the within-laboratory level as a primary attribute of most methods. By the above definition, the determination of bias in the analysis of a material by a new method requires the knowledge of either the true value for the analyte in the sample or the assignment of an accepted reference value. In some cases these values may be known or assigned through the use of fortified or spiked samples, certified reference materials, analysis by another presumably unbiased method, or internal controls. When analyzing fortified or spiked samples, bias is often

expressed by the terms recovery or percent recovery, i.e., test result, divided by the expected (assigned) value for the added spike material, multiplied by 100.

In other instances, there may be no direct means of determining the bias of a new method's test results. Under these circumstances the recovery of the analyte by the new method is sometimes assessed in relation to the results of an accepted reference method and expressed as relative recovery (ISO 2000). If no such reference method is available, relative recovery may be defined by the new method itself. An example of this can be found where the test results from two simultaneously processed and analyzed samples with unknown quantities of analyte are compared. In this case the two test results can be expressed as the ratio of the analyte recovered in the two samples. By designating one of these unknown samples as a reference, the relative recovery of the analyte from any number of additional simultaneously processed and analyzed unknown samples can be determined and compared to each other based on their respective recovery ratios with this reference sample. For methods with high variability, this technique is still useful since the variation can be quantified through measurement of standard deviation. In addition, if the reference sample can be considered to be the same in different test runs of the method, i.e., a consistent material containing the same (albeit unknown) quantity of analyte, the respective recovery ratios with this sample can also be used for comparing the relative recoveries from other unknown samples in these different sets of analyses.

2.4.4 Limit of Detection (LOD)

Limit of detection can be defined as the minimum amount of analyte that can be reliably detected (i.e., distinguishable from known and characterized background with a given level of confidence). The LOD measurements establish a baseline detection value under optimal conditions. Culture based methods are typically expected to be capable of detecting single target organisms in large samples. Where no organisms are detected, the result is reported as <1 organism per sample volume or mass.

2.4.5 Linearity

Linearity is defined as the ability of the analytical procedure to obtain test results within a given range, which are directly proportional to the concentration of analytes (microorganisms or nucleic acid) in the sample.

2.4.6 Calibration and Other Performance Attributes of Absolute Quantitative Test Methods

The performance attributes discussed in Sections 2.4.1 thorugh 2.4.5 are generally relevant to both qualitative and quantitative analytical methods. They can be characterized either directly or with minimal mathematical data transformations on the basis of the response values of the detector or instrument utilized in the method, or in the case of culture based methods, the bacterial/viral counts obtained. For methods specifically designed to obtain absolute quantitative measurements of an analyte, several additional operational limits and performance attributes may require determination and in many instances the relationships between detector or instrument response and analyte concentrations must also be established through the development of a calibration curve using reference standards of known concentration. The concentration of analyte in a reference standard can be established by several alternative approaches including: assignment or certification based on experimental work by some national or international organization; consensus or certification based on collaborative experimental work under the auspices of a scientific or engineering group; or in some cases, assignment based on analysis results of a reputable commercial or other non-commercial establishment. As mentioned in Section 1.4, analytical detection is only a part of the complete method and wherever appropriate, calibration to establish complete method recovery may be necessary.

For standard curve development, the simplest model (e.g., linearity) that adequately describes the concentration-response relationship should be used. The calibration curve is also used to establish the limit of quantification of the method (i.e., the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy) and the quantitative range of the method (i.e., the interval between and including the upper concentration and limit of detection of the analyte over which the concentration-response model provides suitable precision and accuracy). During this process, the statistical dispersion of the standard measurements by the instrument or detector (instrument precision) is combined with the model assumptions to generate a calibration equation. A statistic such as R-squared combined with a confidence interval can be used to provide a measurement of reliability or uncertainty for an estimate from an “unknown” sample based on the calibration curve model.

2.4.7 Special Considerations for Culture-based Microbiological Test Methods

Historically, most microbial work has involved cultivation (viability testing) of microorganisms in a selective medium for enumeration or for the determination of presence or absence of the target microorganism(s). EPA or other regulatory requirements determine whether quantitative or qualitative results are required and may specify which methods are allowed. While not strictly considered as absolute quantification methods, due to the near impossibility of knowing the true amount of viable microorganisms in a sample, quantitative culture-based microbiological methods have performance attributes and operational limits that are the same as, or are analogous to, most of the terms defined above, including specificity, sensitivity, precision, recovery, limit of detection, linearity and limit, and range of quantification (ISO 2000). Unlike chemical analytical methods, however, many of the performance attributes of culture methods are based upon particle distribution (i.e., distribution of the microorganisms) as defined by the Poisson distribution or where over-dispersion occurs (often in environmental waters due to clumping or simply the natural variability of target microbes), using a negative binomial distribution model (ISO 2000). Due to the often skewed distribution of target microbes in waters, when using methods that are based on normality of count data, it is generally necessary to convert the data to log-base 10 (or in other ways transform) to meet basic statistical assumptions when describing metrics such as the mean and standard deviation (Youden and Steiner 1975; ISO 2000; APHA 2005).

Over-dispersion in a culture method can also be caused by a number of technical factors including variability in collection and/or preparation of the starting sample, and its dilution. Other factors such as physico-chemical properties and overall microbial composition of the sample, incubation stress, quality, and origin of detection media components and incubation conditions can also contribute to variability and uncertainty in measurements by these methods.

2.4.8 Other Operational Limits and Quality Control Measures

Analyte stability may have an important effect on the test results of many analytical methods. Consequently, the determination of acceptable holding times and storage conditions of samples and (if applicable) reference standards may be an important operational limit that should be determined during the primary validation process. Parameters that should be investigated using experimental designs are similar to those employed for determining the precision and bias

or relative recovery of the method (see above). The objective is to identify the limits of conditions that produce no significant differences in test results compared with those obtained from analyses of fresh samples at a specified confidence level. Different sample matrices may also affect test results. Thus, a number of sample matrices that are representative of the intended use of the method need to be tested to determine the range and/or proportion of these samples compared to those obtained from a specified reference matrix (i.e., no significant difference at a specified confidence level).

Many analytical methods employ the use of matrix spike or internal control materials as surrogates of the true analyte for the routine determination of the efficiency of extraction from sample matrices and interferences in the analyses. If these quality control materials are utilized in a method, studies should be performed to determine whether their recoveries and responsiveness to interferences is representative of the true analyte. Another important consideration is to ensure that control material does not interfere with the detection of the true analyte at low concentration.

2.5 Writing the Method

The next step in the validation process is to prepare a complete written description of the method. Historically, EPA methods have been written in a format that includes the following components: scope and application; method summary; definitions; interferences; health and safety; equipment and supplies; reagents and standards; sample collection, preservation and storage; quality control; calibration and standardization; procedural steps; calculations and data analysis; method performance; pollution prevention; and waste management. Each component of this format is described in greater detail in Appendix A of this report. Note that this is a recommended format, and not a requirement. In this guidance, we further recommend inclusion of the numerical and descriptive specifications of the method's operational limits and performance attributes determined from the within-laboratory testing results during primary validation. This is justified since for some methods the primary validation process should be sufficient for meeting the intended use of the method. In addition, if subsequent validation of the method in a multi-laboratory collaborative study is needed, the inclusion of the primary validation study specifications in the written method will serve to inform the design and interpretation of results of the collaborative studies and may also be useful for establishing analytical quality control systems.

2.6 Multi-laboratory Validation Studies

EPA has historically recommended the use of interlaboratory collaborative studies for the validation of methods that are expected to see widespread use or to support regulatory activity. A tiered approach developed under the streamlining initiative (U.S. EPA 1996) has typically been used by EPA for the validation of microbiological methods. This approach takes into consideration the level of intended use for a method and as such will minimize the validation requirements of limited-use methods (single-laboratory and single-industry use) and instead focuses resources on validation of methods that are intended for nationwide use. Because QC acceptance criteria are developed from validation studies and validation requirements vary with each tier, the statistical procedures used to develop the criteria will vary by tier as well.

2.6.1 Tier 1 Validation

Tier 1 refers to new methods or method modifications that will be used by a single laboratory for one or more matrix types (i.e., air, water, soil). Validation requirements for Tier 1 have reflected this limited use and typically require single-laboratory testing in the matrix types for which the method will be used. Under Tier 1, single laboratories are able to use modified methods without the burden of conducting an interlaboratory method validation study.

2.6.2 Tier 2 Validation

Tier 2 refers to new methods or method modifications that will be used by multiple laboratories analyzing samples of one matrix type. Validation for Tier 2 typically requires a three-laboratory interlaboratory study using the matrix in which the method will be used.

2.6.3 Tier 3 Validation

Tier 3 refers to new methods or method modifications that will be used by laboratories on a nationwide basis for all matrix types. Validation at Tier 3 requires a nine-laboratory interlaboratory study on nine matrix types. Validation must be performed on a minimum of nine matrix types in each sample for which the method will be applied.

This tiered approach guidance continues in this document with the recognition, however, that decisions concerning the numbers of participating laboratories in different collaborative

studies may be influenced by a number of additional factors. Such factors, as detailed further below, can include the specific objectives of a study including statement of a testable hypothesis, the availability of laboratories that have instrumentation for and experience with the method, the complexity of the method, the underlying statistical theory and subjective judgments. Study design must be peer-reviewed by qualified members of the scientific community including statistician(s) before the study is implemented.

2.6.4 Types of Multi-laboratory Validation Studies

While the ultimate goal of interlaboratory validation is to demonstrate the suitability of an analytical method for its intended purpose under conditions of widespread use, several different types of collaborative studies may be required before making this final assessment. Some methods may require a collaborative study to simply establish an acceptable reference standard or material as one of the steps in this process (Horwitz 1995, Holmes *et al.,* 2001).

Primary Validation or Laboratory Performance Study. Collaborative studies have been developed into a widely used tool for testing performance characteristics of chemical methods, but this process is normally associated with the widespread availability of laboratories that already have extensive experience with these methods. This may not be the case for many newer microbiological methods (ISO 2000). For methods of this type, the more prudent next step in the validation process may be to conduct a collaborative laboratory performance study. Studies of this type are usually conducted with the objective of evaluating or improving laboratory performance of the method. Reported results by different individual analysts or laboratories are compared with those from other laboratories or with the known or assigned reference values of a limited number of test samples with a simple, well-defined matrix. These studies may also allow for assessments of method robustness, the clarity of the test procedures provided in the written method, and more generally, whether the method appears ready for widespread implementation. More than one study of this type may be required if correctable deficiencies related to any of the above aspects of the method are discovered. This type of study may also be called primary validation. These studies should not be confused with laboratory proficiency testing, which is normally associated with the evaluation or accreditation of laboratories in performing a previously validated and accepted method.

Secondary Validation or Method Performance Study. The type of collaborative study that is normally conducted in the validation process is a method performance study. The objective of these studies is normally to provide specifications for the methods performance attributes across a representative sample population of laboratories that would be expected to employ the method for its intended purpose. A second or alternative objective may be to demonstrate equivalency of a new method with an established reference method. This type of study is also sometimes referred to as secondary validation and guidelines on the experimental and statistical design of; preparation for; and implementation and analyses of results of studies of this nature have been published by a number of organizations (ISO, AOAC, ASTM, IUPAC, FDA, NCCLS, and others). A synopsis of recommendations that generally are consistent in these different guidance documents and other recommendations that may be specifically associated with the validation of microbiological test methods are provided in the following sections.

2.6.5 Participating Laboratories

The decision on how many laboratories to include in a method performance study often takes into account the effect of sample size on the estimation of confidence intervals for the performance parameters being tested. These intervals can be predicted based on statistical theory for different numbers of test results from different laboratories (Youden and Steiner 1975, AOAC 1989, ISO1994a).

A relationship exists between the number of participating laboratories, the number of test samples, the acceptable margin of error (precision), and the desired level of confidence when considering the number of independent laboratories required for the validation of a method. There are a number of documents (McClure, 1990) that discuss these relationships for quantitative method validation, however, there is very little guidance regarding qualitative methods. In general, binomial data sets (e.g., presence/absence) generated from qualitative methods require more independent laboratories and test samples to achieve equivalent levels of margin of error and confidence levels when compared to quantitative data. Following is a mathematical model to estimate the margin of error (p_2) (precision) given a certain number of laboratories (L) and test samples (m) for a method that is required to correctly classify at least

(80%) (p_1) of the positive test portions (sensitivity rate), with a confidence limit of 90%. The equation is as follows:

$$p_2 = 100\, Z_\alpha\, \hat{S}_a / [L^{1/2} m\, (p_1/100)] \qquad \text{Equation (5)}$$

Where:

L = 2, 3….., 25
m = 3, 5, 10
α = .05 (corresponds to Z_α = 1.645 or 90% confidence limit)
$\hat{S}_a$ = estimated standard deviation (simulation method) of the number of positive responses per laboratory and 80% sensitivity rate to estimate the margin of error (see Figure 2).

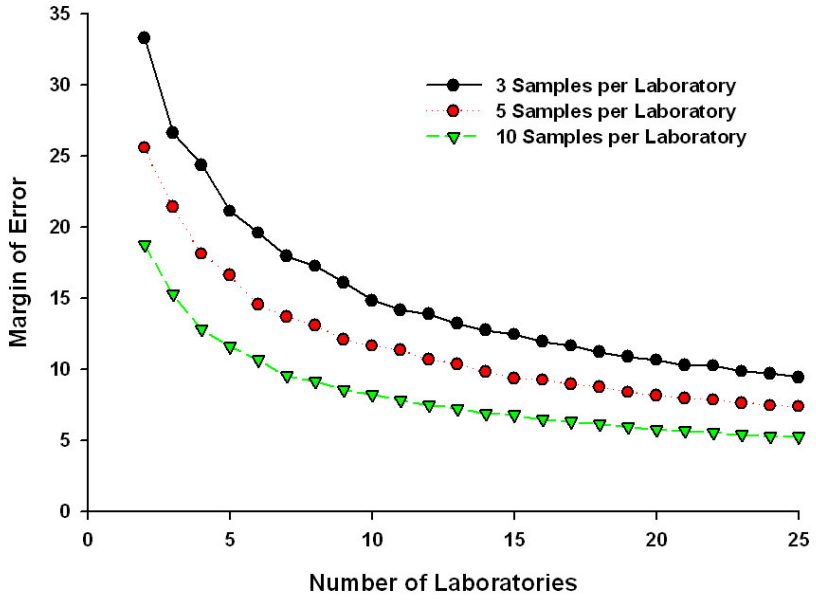

Figure 2. Margin of Error for a Method with 80 % Sensitivity Rate and Confidence Interval of 90 %

Because of the diminishing return in decreasing uncertainty that is associated with increasing numbers of independent test results from different laboratories, a common compromise is 8-15 laboratories to participate. The expectation is that a small number of these data sets may be statistical outliers or may be obtained from incorrect performance of the method and thus will be excluded. However, there are limitations to applying this purely statistically based approach to microbiological methods. Ultimately decisions on sample size (i.e., number of

laboratories) in a study must be based to some degree on a subjective judgment as to whether the results of the study will be effective in demonstrating method suitability (ISO 2000). Method complexity and the resource availability may also be important factors in making this decision.

It is generally recommended that the selection of laboratories for a method performance study not be restricted to only laboratories with extensive experience with the specific method under consideration. Conversely, it is highly recommended that all participating laboratories have extensive experience with the techniques and/or instrumentation utilized in the method. Criteria for selection should include precision and accuracy of the laboratory when performing the technique on blind samples (rsd). For newer methods, it has been suggested that the performance attribute specifications obtained in these studies be reported as preliminary or initial estimates because they could further improve as laboratories gain experience with the method (ISO 1994a).

2.6.6 Test Materials

Test materials in a validation study have been previously defined as specific analyte/matrix/concentration combinations to which the method performance parameters apply (Horwitz 1995). Similar to within-lab method validation studies, the test materials used for collaborative method performance studies should be representative of the range of matrices intended for analysis by the method. In most cases natural samples of the materials containing naturally occurring analytes are preferred. Several different analyte concentrations should be tested and the range of analyte concentrations should be within the operational range of the method as specified from primary validation study results and should also be representative of the expected range of concentrations associated with the intended use of the method. If a specific concentration of analyte is known or predicted to be associated with a regulatory requirement, the concentrations tested should include and bracket this value. Larger numbers of test materials are generally required for method equivalency studies (ISO 2004).

A highly important prerequisite of these studies is that the test samples that are analyzed by the different laboratories should originate from materials in which the analyte is homogeneously distributed. This is often challenging with microbes. This property can be established by the use of certified reference materials or determined from analyses of replicate samples with materials in the laboratory that procures them or prepared by a reference

laboratory. In the latter instances, precision estimates from these analyses should be compared with the method's primary validation study precision estimates under repeatability conditions. It is important to remember that in the case of most microbiological methods, this property will be based upon particle distribution (i.e., distribution of the microorganisms) unlike in chemical methods. If this property can not be established with reasonable certainty in natural materials or if natural materials can not be readily found that contain the analyte, then another commonly employed alternative is the utilization of spiked materials. For laboratory prepared spikes, documentation of how precise the spikes are will be useful. Homogeneity of the analyte in the spiked materials should also be confirmed as above. If analyte recovery is a performance attribute that is being evaluated, spike levels should be sufficiently high to be readily differentiable from the levels of any naturally occurring analytes in the materials based on the method's primary validation study precision estimates under reproducibility conditions.

2.6.7 Replication of Test Materials

Precision and bias (or recovery) are the performance attributes that are most commonly characterized in collaborative studies of quantitative methods. Accuracy as defined by ISO (ISO 1994a) is influenced by both of these parameters and precision in turn is influenced by both between-lab repeatability and reproducibility components. As shown above, the number of replicate test results per test material will influence the uncertainty of estimates for repeatability and reproducibility standard deviations. The latter uncertainty value is also shown to be a function of the ratio of these two standard deviations. It is commonly observed with many chemical analytical methods that this ratio is relatively high, i.e., the variability of test results obtained under repeatability conditions is considerably smaller than that obtained under reproducibility conditions (AOAC 1989). When this situation occurs, analyses of more than two replicate samples of the different study materials will provide relatively little additional information. Microbiological analytical methods may differ in this regard, however, and analysis of additional replicate samples is warranted. Repeatability and reproducibility precision specifications generated from the primary validation study or results from collaborative laboratory performance studies can be used to determine the most appropriate number of replicate test sample analyses to perform. While the use of known replicate samples is a common

practice, the use of blind replicate samples is generally recommended to eliminate the potential for any selectivity by the laboratories in their procedures and reporting of test results.

2.7 Development of a Study Plan and Study Implementation

Once the study design has been determined it should be documented in a study plan that provides a detailed description of the study's objectives and the clear statement of a testable hypothesis(es). These objectives should include management and implementation of the study; establishment of requirements for data reporting; documentation and review by laboratories; means for assessment of adherence to instructed validation methodologies, accuracy, and completeness; and specific instructions for data analysis including statistical tests and anticipated metrics for the results. Any study-specific procedural instructions that are not fully explained in the written method (e.g., sample storage conditions, when to perform analyses, and how to record and report the data) should be written in detail and be provided with the method to the laboratories well in advance of the study. New study organizers can also consult study plans for previously validated EPA methods or from the general scientific literature, if applicable. An example of study plan for a previously conducted validation study by EPA is given in Appendix B. Additional guidance that may be useful in the study planning and implementation process can be found in documents from several other organizations (AOAC 1989, ISO 2000, ASTM 2001).

2.8 Reporting Results

Results from the method performance study should be disseminated and made available to the scientific community for peer review before presentation at a scientific meeting, peer-reviewed publication, and/or publication of a technical manual. Studies completed for the purposes of method approval (via publication in a final ruling) in the Federal Register may fulfill this requirement by inclusion in the docket for the proposal of the method.

The report should identify the participant laboratories and describe all test materials together with how they were collected and/or prepared. It should also describe all reagents, spiking materials and reference standards used in the study, including their commercial or organizational sources, if applicable. If any of these materials were custom-prepared for the study, the report should include a description of how they were prepared and documentation of

their homogeneity. In many instances (e.g., when submitting for peer-reviewed publication), the report will also provide a summary of the study design, procedures and statistical methods used for analyses of the results.

A critical component of the report is a presentation and discussion of the numerical and descriptive specifications for the performance attributes of the methods that were determined from the collaborative study. These specifications summarize the performance of the method under the specified conditions of the study and are the primary source of information for determining the suitability of the method for its intended purpose in the context of widespread use. The report may also include the development of quantitative QC criteria for initial and ongoing method/laboratory performance assessments. Lastly, if possible, a statistical assessment of the method's comparability with any available reference method should be provided. Criteria and guidelines for the establishment of equivalence between microbiological test methods have been developed (ISO 2004).

3.0 REFERENCES

AOAC. 1989. Guidelines for collaborative study of procedure to validate characteristics of a method of analysis. *J. Assoc. Off. Anal. Chem*. **72**, 694–704.

AOAC. 2006. AOAC Task Force Recommends Best Practices for Validation of Microbiological Methods; Final Report Submitted. Inside Laboratory Management. November/December p 10.

American Public Health Association (APHA) 1998. Standard Methods for the Examination of Water and Wastewater (20th Ed,) 922B.6.c.

APHA, 2005. Part 9020. Quality Assurance/Quality Control. *In* Standard Methods for the Examination of Water and Waste Water, http://standardmethods.org/store/BrowseSM.cfm.

ASTM, Designation E691-99, "Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method," *Annual Book of ASTM Standards,* Vol. 14.02, ASTM, West Conshohocken, PA, 2001, Section 17.1.

Centers for Disease Control and Prevention and National Institutes of Health, 2007. Biosafety in Microbiological and Biomedical Laboratories, 5th edition. U.S. Department of Health and Human Services. U.S. Government Printing Office, Washington, D.C.

Deep, R. 2006. Probability and Statistics. Academic Press, New York.

Dols, T and B. Ambrecht, 1976. Simplex optimization as a step in method development. *J. Assoc. Off. Anal. Chem*. 59: 1204-1207.

Fleming, D.O. and Hunt, D.L. eds. 2000. Biological Safety: Principles and Practices. ASM Press, Washington, D.C.

Green, J.M. 1996. A Practical Guide to Analytical Method Validation. Analytical Chemistry 68:305A-309A.

Holmes H, C. Davis, A. Heath, I. Hewlett, N. Lelie. 2001. An international collaborative study to establish the 1st international standard for HIV-1 RNA for use in nucleic acid-based techniques. J. Virol. Methods 92:141-150.

ICH. 1996. Validation of analytical procedures: Methodology ICH-Q2B. International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use.

International Organization for Standardization. 1994a. ISO 5725-1:1994. Accuracy (trueness and precision) of measurement methods and results - Part 1: General principles and definitions. Geneva, Switzerland.

International Organization for Standardization. 1994b. ISO 5725-2:1994. Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method. Geneva, Switzerland.

International Organization for Standardization. 1994c. ISO 5725-3:1994. Accuracy (trueness and precision) of measurement methods and results - Part 3: Intermediate measures of the precision of a standard measurement method. Geneva, Switzerland.

International Organization for Standardization. 2000. ISO/TR 13843:2000(E). Water quality – Guidance on validation of microbiological methods. Geneva, Switzerland.

International Organization for Standardization. 2004. ISO 17994:2004. Water quality - Criteria for establishing equivalence between microbiological methods. Geneva, Switzerland.

McClure F.D. 1990. Design and analysis of Qualitative Collaborative Studies: Minimum Collaborative Program, J. Assoc. Off. Anal. Chem. Vol. 73, No. 6.

Niemela, S. I. 2003. Uncertainty of quantitative determinations derived by cultivation of microorganisms. Centre for Metrology and Accreditation, Helsinki, Finland.

USEPA. 1992. Consensus Method for Determining Groundwaters Under the Direct Influence of Surface Water Using Microscopic Particulate analysis (MPA). EPA 910/9-92-029.

USEPA. 1996. Guide to Method Flexibility and Approval of EPA Water Methods. EPA 821D96004.

USEPA. 2004. Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples EPA 815-B-04-001. United States Environmental Protection Agency.

USEPA. 2005. Manual for the Certification of laboratories Analyzing Drinking Water. EPA 815-R-05-004.

US FDA, *Center for Drug Evaluation and Research (CDER). 2001*. Guidance for industry. Bioanalytical method validation. *http://www.fda.gov/cder/guidance/index.htm*

Youden and Steiner, 1975. Statistical Manual of the Association of the AOAC, Association of Official Analytical Chemists. Arlington, VA.

Appendix A – Environmental Monitoring Management Council (EMMC) Format for Writing a Method

The components of the Environmental Monitoring Management Council (EMMC) format for writing a method are detailed below:

1.0 Scope and Application

Include the matrices to which the method applies, a generic description of method sensitivity and the data quality objectives which the method is designed to meet. Much of this material may be presented in a tabular format.

2.0 Summary of Method

Summarize the method in a few paragraphs. The purpose of the summary is to provide a succinct overview of the technique to aid the reviewer or data user in evaluating the method and the data. List sample volume, concentration, other preparation steps employed, the analytical instrumentation, and the techniques used for quantitative determinations.

3.0 Definitions of Method

Include the definitions of all method-specific terms here. For extensive lists of definitions, this section may simply refer to a glossary attached at the end of the method document.

4.0 Interferences

This section should discuss any known interferences, especially those that are specific to the performance-based method. If known interferences in the reference method are not interferences in the performance-based method, this should be clearly stated.

5.0 Safety

X Above and beyond good laboratory practices

X Disclaimer statement (look at ASTM disclaimer)

X Special precautions

X Specific toxicity of reagents

X Not appropriate for general safety statements

This section should discuss only those safety issues specific to the method and beyond the scope of routine laboratory practices. Reagents that pose specific toxicity or safety issues should be addressed in this section.

6.0 Equipment and Supplies

Use generic language wherever possible. However, for specific equipment do not assume equivalency of equipment that was not specifically evaluated, and clearly state what equipment and supplies were tested (i.e. incubators, water baths).

7.0 Reagents and Standards

Provide sufficient details on the concentration and preparation of reagents and standards to allow the work to be duplicated, but avoid lengthy discussions of common procedures.

8.0 Sample Collection, Preservation and Storage

Provide information on sample collection, preservation, shipment, and storage conditions.

9.0 Quality Control

Describe specific quality control steps, including such procedures as laboratory control samples, QC check samples, instrument checks, etc., defining all terms in Section 2.0.

10.0 Calibration and Standardization

Discuss initial calibration procedures here. Indicate frequency of such calibrations, refer to performance specifications, and indicate corrective actions that must be taken when performance specifications are not met. This Section may also include procedures for calibration verification or continuing calibration.

11.0 Analytical Procedure

Provide a general description of the sample processing and instrumental analysis steps. Discuss those steps essential to the process, and avoid unnecessarily restrictive instructions.

12.0 Calculations and Data Analysis

Describe qualitative and quantitative aspects of the method. List identification criteria used. Provide equations used to derive final sample results from typical instrument data. Provide discussion of estimating detection limits, if appropriate.

13.0 Method Performance

A precision/bias statement should be incorporated in the Section, including:

- X Detection limits
- X Source/limitations of data

Provide detailed description of method performance, including data on precision, bias, detection limits (including the method by which they were determined and matrices to which they apply), statistical procedures used to develop performance specification, etc. Where performance is tested relative to the reference method, provide a side-by-side comparison of performance versus reference method specifications.

14.0 Pollution Prevention

Describe aspects of this method that minimize reagents or prevent pollution that may be attributable to the reference method.

15.0 Waste Management

Cite how waste and samples are minimized and properly disposed.

16.0 References

17.0 Tables, Diagrams, Flowcharts, and Validation Data

Appendix B – Interlaboratory Collaborative Study

Interlaboratory Collaborative Study

Validation Protocol for the Quantitative Three Step Method

Prepared by:
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TABLE OF CONTENTS

SECTION		PAGE
1.	INTRODUCTION	3
2.	COLLABORATORS	5
3.	STUDY DESIGN	7
4.	TEST SAMPLE PREPARATION	11
5.	QUALITY ASSURANCE	12
6.	REFERENCE METHOD – Revised AOAC Method 966.04	14
7.	QUANTITATIVE THREE STEP METHOD	23
8.	REPORTING RAW DATA	36
9.	STATISTICAL ANALYSIS	36
10.	REFERENCES	39
11.	LIST OF APPENDICES	40

A. EPA Quality Assurance Project Plan
B. AOAC Method Data Sheets
C. TSM Data Sheets
D. Media and Reagent Preparation Sheets
E. Parameters for Testing Chemicals
F. TSM Log Reduction Plan
G. Material Safety Data Sheets (MSDS)
H. Safety Checklist

Section 1. INTRODUCTION

In response to the intentional release of spores of *Bacillus anthracis* in 2001, and the associated need for determining the performance of sporicidal and sterilant chemicals for use in the decontamination of buildings, the U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) initiated a research program to evaluate and improve laboratory efficacy test methods for sporicides and sterilants. Due to limitations and deficiencies associated with the Agency's reference method for efficacy testing, AOAC Method **966.04**, a key priority related to EPA's Homeland Security efforts has been the development of a quantitative methodology to replace or augment Method **966.04,** with emphasis on the regulatory aspects of verifying the performance of chemicals used in building decontamination. The most significant concerns associated with Method **966.04** are the qualitative nature of the method, the use of raw garden soil extract as a source of minerals for spore production, the carrier materials (unglazed porcelain and silk suture loops), the lack of a standardized procedure for enumeration of spores, spore wash-off, and the long incubation time (21 days). Several of the deficiencies were recently addressed by EPA in a collaborative study to modify the AOAC method.

In a pre-collaborative study, two quantitative carrier-based test methods for determining the efficacy of liquid sporicides and sterilants on a hard surface, the Standard Quantitative Carrier Test Method – American Society for Testing and Materials (ASTM) E 2111-00 and an adaptation of a quantitative micro-method (the Three Step Method) as reported by Sagripanti and Bonifacino, were compared (1). By conducting the study in a comparative, standardized manner across multiple laboratories, valuable statistical information on the performance of two quantitative methods was generated. Based on the statistical parameters measured and the additional test method attributes related to logistics and human resources, the Three Step Method (TSM) was determined to be the more suitable method, and will be advanced to the AOAC validation process. The flexibility in the TSM for incorporating coupon materials other than glass was considered as an advantage, and that the practice time and training issues (identified by analysts who performed the method) could be resolved through the re-organization of the protocol and the use of a flowchart and process checklist. As the development and use of quantitative test methods for sporicides and sterilants proceeds, additional studies will be necessary to develop meaningful and relevant performance standards (i.e., pass/fail criteria with a minimum LR).

Although the details of the method have been published, the TSM is considered a new method (i.e., for the purpose of this initiative) with a limited amount of historical use in the regulatory arena. The TSM is a quantitative procedure for hard surfaces – considered as a significant advantage over qualitative procedures and suspension-based tests. The TSM uses 5 × 5 × 1 mm glass coupons to deliver spores into the sporicidal agent (400 µL) contained in 1.5 mL microcentrifuge tubes, 3 coupons per chemical treatment. Following exposure to the test chemical and neutralization, spores are removed from the carriers in three fractions by sonication and vortex-mixing. Liquid from each fraction is plated on recovery medium for viable spore enumeration. Control counts (water control) are compared to the treated counts and the level of efficacy is determined by calculating the Log_{10} reduction (LR) of spores; LR = log_{10} (mean

spores/control carrier) - $\log_{10}$ (mean spores/treated carrier). The original procedures for the TSM were reported by Sagripanti and Bonifacino (2). However, during the course of the pre-collaborative studies, EPA refined and altered the method slightly, and finalized the protocol for the purpose of this validation study. It should be noted that following the completion of the pre-collaborative study, a similar version of the TSM was accepted and published by ASTM International (3).

The purpose of this Interlaboratory Collaborative Study (CS) is to evaluate the TSM according to AOACI Official Methods of Analysis (OMA) procedures for official method validation. The applicability will be limited to one spore-forming microorganism (*Bacillus subtilis*), one hard surface (glass), liquid formulations of sporicides and sterilants, and without organic burden added to the spore inoculum. The test chemicals used in the CS represent three chemical classes of sporicides: sodium hypochlorite, a combination of peracetic acid and hydrogen peroxide, and glutaradehyde. The suitability of the TSM for porous materials and gaseous formulations will require additional collaborative studies. If the TSM is validated for *B. subtilis*, the Study Director will propose language in the validation report to allow the testing of other *Bacillus* species, such as *B. anthracis*, with the TSM.

The overall objective of the CS is to evaluate the performance of the TSM by generating and comparing control counts and efficacy data and assessing the degree and source(s) of variability associated with the data, both within and between laboratories, when the method is used in actual practice. AOAC Method **966.04**, Sporicidal Activity of Disinfectants, is recognized as the reference method. Method **966.04** provides a qualitative measure of product efficacy against spores of *B. subtilis* and *Clostridium sporogenes* dried on two types of carriers, porcelain penicylinders and silk suture loops. Sixty-carrier tests on three lots of product are required for an EPA registration – all carriers must show no growth to support a sporicidal claim. For the purpose of this CS, only the *Bacillus* and hard surface (porcelain penicylinders) components of Method **966.04** will be evaluated. The comparative testing with the reference method is problematic due to its qualitative nature. In this study, chemical treatments from one of three replications per product will be tested using both the reference method and TSM. The cost of conducting Method **966.04** is a limiting factor and it would not be feasible to generate enough inoculated porcelain carriers to do each treatment and replication.

Prior to initiation of this collaborative effort, the AOAC INTERNATIONAL will assemble a review panel, the AOAC Sporicidal Method Expert Review Panel (ERP), to evaluate the CS protocol. The ERP, along with members of the AOAC INTERNATIONAL Methods Committee on Microbiology, will be engaged early in the development of the study design to ensure the protocol format and test design are acceptable and meet the goals established for the AOACI Official Methods process.

Section 2. COLLABORATORS

A total of eight to eleven laboratories will participate in this study. The laboratories selected will have existing microbiology programs, appropriately trained personnel, and have the capability of conducting the validation protocol within the timeframe established by the Study Director. The OPP Microbiology Laboratory is the lead laboratory. Two Food and Drug Administration (FDA) laboratories are currently collaborating with EPA per an Interagency Agreement (IAG) and will also participate in the study. A third FDA laboratory is expected to participate under the IAG. At least five laboratories who have not been involved with the development or evaluation of the TSM will be included. The name and associated contact person for each participating laboratory are provided in Table 1.

Table 1. List of collaborators

Lab Number	Laboratory Name	Contact Information
1	U.S. EPA OPP Microbiology Laboratory	**Stephen Tomasino**, Ph.D., Study Director **Rebecca Fiumara**, Co-Study Director U.S. EPA Environmental Science Center 701 Mapes Road, Ft. Meade, MD 20755-5350 Phone for S. Tomasino: 410-305-2976 Phone for R. Fiumara: 410-305-2635 FAX: 410-305-3094 Email: tomasino.stephen@epa.gov
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4	U.S. FDA Office of Science and Engineering Laboratory (OSEL) - White Oak Campus	**Victoria Hitchins,** Ph.D. U.S. FDA Office of Science and Engineering Laboratories Center for Devices and Radiological Health 10903 New Hampshire Avenue Silver Spring, MD 20993 Phone: 301-796-0258 Email: victoria.hitchins@fda.hhs.gov

5	Advanced Sterilization Microbiology Laboratory	**Harriet Chan-Myers** Advanced Sterilization Microbiology Laboratory 33 Technology Drive, Irvine, CA 92618 Phone: 949-453-6330
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9	Bioscience Labs	**Daryl Paulson** BioScience Laboratories Inc. 300 N. Wilson Ave, Suite 1, Bozeman, MT 59715 Phone (406) 587-5735 Fax (406) 586-7930 Email: dpaulson@biosciencelabs.com
10	Ohio Department of Agriculture	**Jim Agin** Ohio Department of Agriculture Bldg 3, Consumer Analytical Lab 8995 E Main St, Reynoldsburg, OH 43068 Work Phone: 614-728-0198 Fax: (614) 728-6322 Email: agin@odant.agri.state.oh.us

Section 3. STUDY DESIGN

A. The Study Director, Dr. Stephen Tomasino, is responsible for organizing the CS and assessing the preparedness of each collaborating laboratory prior to initiation of research. Toward that effort, the Study Director and the OPP Microbiology Laboratory Quality Assurance Unit will conduct a readiness review of each participating laboratory to ensure compliance with EPA's Quality Assurance Project Plan 2003-01 (Appendix A). The Co-Study Director, Rebecca Fiumara, will serve as the technical lead for the TSM.

B. The method protocols, standardized test forms and data sheets (Appendices B and C), media preparation sheets (Appendix D), selected media and reagents, test chemicals, and inoculated porcelain penicylinders will be provided by the Study Director. Test parameters for each chemical treatment describing the conditions for testing (e.g., dilution, neutralizer, contact time, temperature, etc.) will be provided to each laboratory by the Study Director (Appendix E).

C. The Study Director recognizes that it is desirable to distribute the chemical treatments to the testing laboratories in containers that are marked only with a treatment code. However, due to the instability of test chemicals such as diluted sodium hypochlorite, it will be necessary for each lab to prepare the actual test formulations (i.e., perform dilutions) on-site. The test chemicals will be provided by the Study Director. The test scheme will be randomized to account for potential subjectivity by the analysts.

D. In order to have a balanced design that will be conducive to a straight-forward statistical analysis, the labs will be asked to perform the same number of tests per day. The study design calls for testing three chemicals, three levels each (high, medium and low), one chemical per day. Three replications are required. Assuming no repeat testing, 9 test days will be required to complete the CS. Water controls (control carrier counts) will be included each test day for the TSM. Each treatment will be evaluated by the TSM; however, Method **966.04** will be conducted concurrently on the first replication only for a total of nine 30-carrier AOAC tests per laboratory.

E. Testing must be initiated within 4 weeks after the readiness review, practice/training and receiving supplies, and must be completed within 12-14 weeks after initiation of the first test.

F. Two or three technicians will be required per test day for approximately 6 hours. Each laboratory will be encouraged to establish a technician team which will conduct all tests, i.e., it is important to design the study so that the differences among technician teams do not affect the outcome. If the same technician team always conducted the testing, the technician effect will not confound the results. Practice runs will be encouraged in advance of testing to ensure analyst proficiency in performing each method.

G. It is important to randomize any steps in which subjective decisions or unknown factors could affect the conclusions. The experimental results could be criticized because the order of experimentation was systematic; any systematic trend in environmental conditions would affect the results. It is preferable to have a numbering system or positioning arrangement that gives each item a unique identification. In this study, the Study Director will provide the randomized order of testing of chemicals for each lab using an acceptable method of randomization. The randomization will be done before the experimentation is initiated.

H. Test chemicals used in the study are:

1. Sodium hypochlorite (reagent grade, Sigma-Aldrich sodium hypochlorite solution, 12% available chlorine)
2. A combination of peracetic acid and hydrogen peroxide (Spor-Klenz Ready to Use, an EPA-registered commercial sporicide)
3. 2.6% glutaraldehyde (Metricide 14-Day, a commercially available sterilant).

For the purpose of this CS, the test chemicals are experimental components only and are not being tested to support or verify product label claims. Each chemical will be tested at three levels: high (efficacious), medium (moderate efficacy) and low (non-efficacious), to provide a range of efficacy. The test conditions used to generate the range are shown in Table 2. Efficacy data generated by EPA to support the test conditions are presented in Appendix F.

I. **Table 2. Test chemicals and conditions for testing**

Test Chemical	**Treatment Level and Test Parameters***		
	High (LR ≥ 6)	**Medium (LR 2-6)**	**Low (LR 0-2)**
1) Sodium hypochlorite	▪6000 ± 300ppm ▪adjusted pH (7±0.5) ▪30 ± 1 min	▪6000 ± 300ppm ▪unadjusted pH ▪10 min ± 10 sec	▪3000 ± 300ppm ▪unadjusted pH ▪10 min ± 10 sec
2) 0.08% peracetic acid and 1.0% hydrogen peroxide	▪30 ± 1 min	▪10 min ± 10 sec	▪1 min ± 5 sec
3) 2.6% glutaraldehyde	▪180 ± 3 min	▪60 ± 1 min	▪10 min ± 10 sec

*Test conditions expected to generate three levels of efficacy when tested with the TSM.

J. Test chemicals and the Material Safety Data Sheets (Appendix G) will be provided to each laboratory by the Study Director. For sodium hypochlorite treatments, each laboratory will prepare the diluted test chemical from a reagent-grade product per the preparation sheets provided by the Study Director – HACH Test Kits will be used to verify available chlorine for diluted samples. The Study Director will request the use of a single lot for each test chemical. In advance of testing, an analysis of formulation chemistry will be performed on each lot (one container) by OPP chemists to confirm the percent active ingredient.

K. The test microbe is *Bacillus subtilis* (ATCC # 19659) obtained directly from a reputable supplier (e.g., ATCC). Each lab will initiate a new stock culture. Note: An existing stock culture may be used if it meets the quality control standards (e.g., proper documentation, confirmation testing) – Study Director approval is required in this case.

L. Presque Isle Cultures, 3804 West Lake Rd, Erie, PA 16505 will provide inoculated porcelain carriers (approx. 4,000 total for the entire study) to each laboratory per the modified methodology for AOAC Method **966.04.** The vendor, per instructions provided by the Study Director, will follow the revised method (i.e., the use of nutrient agar amended with manganese sulfate) for generation of spore suspensions and inoculation of carriers. No organic burden will be added to the spore inoculum. Carrier counts and HCl resistance will be determined by the vendor, and must meet AOAC method specifications, in advance of shipping the carriers to the collaborative laboratories. A minimum of 1.0×10^5 ($\log_{10}$ density = 5.0) and a maximum of approximately 1.0×10^6 spores/carrier will be required. Multiple lots of inoculated carriers are anticipated.

M. Spores for use in the TSM will be produced by each collaborating laboratory per the method provided. The mean target carrier load for the TSM is 1.0×10^7 spores/carrier or 7.0 logs per carrier – a level suitable for measuring a log reduction of $\geq$ 6 logs. No organic burden will be added to the spore inoculum. Carriers will be inoculated from one spore preparation per laboratory; enough carriers will be inoculated to perform the entire study (approx. 150).

N. The basic CS design:

- 8-11 laboratories
- One microbe
- Three chemicals, each with three levels of efficacy
- Water control for TSM (control carriers)
- Three replications for TSM, one replication for AOAC method
- One carrier type for the TSM (glass)
- One carrier type for the AOAC method (porcelain)
- TSM uses 3 carriers per treatment
- Each AOAC test will use 30 carriers
- Target carrier counts established for each method

O. Example of a test scheme for one laboratory with test chemicals randomized for three replications – see Table 3.

Table 3. Example of the test scheme

Replication	Treatment and Levels	Test Method Performed	
Rep 1 (Day 1)	1. **Sodium Hypochlorite**	TSM*	AOAC 966.04**
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	Yes Yes Yes No
Rep 1 (Day 2)	2. **Peracetic acid/hydrogen peroxide**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	Yes Yes Yes No
Rep 1 (Day 3)	3**. Glutaraldehyde**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	Yes Yes Yes No
Rep 2 (Day 4)	2. **Peracetic acid/hydrogen peroxide**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	No No No No
Rep 2 (Day 5)	3. **Glutaraldehyde**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	No No No No
Rep 2 (Day 6)	1. **Sodium Hypochlorite**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	No No No No
Rep 3 (Day 7)	3. **Glutaraldehyde**	TSM	AOAC 966.04

Table 3. Example of the test scheme (Continued)

Replication	Treatment and Levels	Test Method Performed	
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	No No No No
Rep 3 (Day 8)	1. **Sodium Hypochlorite**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	No No No No
Rep 3 (Day 9)	2. **Peracetic acid/hydrogen peroxide**	TSM	AOAC 966.04
	1. High 2. Medium 3. Low 4. Water Control	Yes Yes Yes Yes	No No No No

*Three carriers per treatment; **30-carriers per treatment

P. **Table 4. Randomized Order of Testing (based on 10 labs)**

Rep*	Random Order of Test Chemicals**									
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10
Rep 1	2, 3, 1	1, 3, 2	2, 3, 1	1, 2, 3	2, 3, 1	2, 1, 3	1, 2, 3	2, 3, 1	1, 2, 3	3, 2, 1
Rep 2	1, 3, 2	3, 1, 2	2, 3, 1	3, 2, 1	1, 2, 3	1, 3, 2	3, 2, 1	1, 2, 3	1, 3, 2	1, 2, 3
Rep 3	2, 3, 1	2, 3, 1	3, 2, 1	2, 3, 1	1, 3, 2	3, 2, 1	1, 2, 3	3, 2, 1	3, 2, 1	2, 3, 1

*Three total tests days per replication; one chemical class tested per day

**1 = sodium hypochlorite, 2 = hydrogen peroxide/peracetic acid, and 3 = glutaraldehyde; order within a test day will be High, Medium, Low, and Water Control

Section 4. Test Sample Preparation

Prepare test chemicals as follows:

1. Sodium hypochlorite (Sigma Aldrich reagent grade solution, approx. 12% available chlorine, product no. 425044), diluted to 6000 ± 300ppm with reagent grade water, pH adjusted (pH 7.0 ± 0.5) with 5% acetic acid. pH adjusted treatment must be used in test within approximately 120 min after preparation; used for the high treatment.

2. Sodium hypochlorite (Sigma Aldrich reagent grade solution), diluted to 6000 ± 300ppm with reagent grade water, unadjusted pH (pH ~10.0). Test within approximately 120 min after preparation; used for medium treatment.

3. Sodium hypochlorite (Sigma Aldrich reagent grade solution), diluted to 3000 ± 300ppm with reagent grade water, unadjusted pH (pH ~10.0). Test within approximately 120 min after preparation; used for low treatment.

4. 0.08% peracetic acid/1.0% hydrogen peroxide product; ready to use product, must initiate testing within approximately 3 hr after dispensing.

5. 2.6% glutaraldehyde product, activate according to product directions, 14-day shelf-life post activation. The activated product may used for testing during the 14-day period.

Section 5. Quality Assurance

The sponsor of this project is the EPA Office of Research and Development. Document archiving will be adequate to ensure that all studies are supported by complete, accurate, consistent, and chronological records from initial collection of raw data to final analysis interpretation and reporting of results.

The preparedness of each laboratory will be assessed by the Study Director and Quality Assurance Unit (QAU) for each laboratory prior to initiation of the study to ensure compliance with a project-specific EPA Quality Assurance Project Plan (Appendix A). The expected level of quality assurance is consistent with EPA Good Laboratory Practices. Numerous guidance documents, standard methods and Standard Operating Procedures (SOPs) will be used to maintain data quality. Proper record keeping and archiving will be performed to ensure the defensibility and reconstructibility or reanalysis of the study. No specific certification is required for this study; however, staff performing the assays must be familiar with standard microbiological techniques such as aseptic transfer, serial dilutions, plate counts and microbe identification. Scientists and analysts involved in testing shall be familiar with each efficacy method and associated procedure (e.g., carrier counts, neutralization confirmation) and will be proficient in conducting each designated efficacy test method.

In-house practice sessions will be required for each laboratory to build proficiency with each method prior to official testing. The Lead laboratory (OPP Microbiology Laboratory) will conduct a series of conference calls with the participating laboratories to discuss the details of each method protocol. Documentation of practice and training for projects will be maintained in a training file for each participating scientist or analyst.

Research documentation shall include project identification, data, and researcher. Pre-printed forms will be used. Research documentation shall be in ink and the use of a single line for correcting entries with the date and initials of the person making the correction and the reason for the change. Project-specific documentation shall be maintained in a project file, or the project file must identify where the documents are stored. Where possible, EPA SOPs and standard forms shall be used for those operations which have become or will become routine, including test methodology, analytical procedures and calibration procedures. SOPs that are comparable to EPA's may be used; however, this will require concurrence by the Study Director or the EPA Quality Assurance Unit. The purpose of the SOPs is to facilitate the uniform

performance of routine procedures. The Standard Operating Procedures for quality control activities are located at: http://www.epa.gov/oppbead1/methods/atmpa2z.htm. Selected electronic spreadsheets and email will be considered as official documentation and will be maintained and archived accordingly.

All preparations of test chemicals, media and reagents will be tracked using an assigned media preparation number. Samples, test chemicals, will be maintained to ensure their integrity. Test chemicals will be stored away from standards, media, and reagents to prevent cross-contamination. No official chain of custody documentation will be required for test chemicals evaluated in this research; however specific information on source, identification, and volume received will be maintained and archived for all test chemicals. All supplies and materials considered "critical" to the quality of the research such as media, reagents, carriers, and test chemicals shall be inspected prior to use to ensure that the shipment has not been damaged or compromised in any way. For pre-sterilized lab supplies, the manufacturer's statement of sterility is acceptable for quality control documentation for sterility; no further testing is required. For growth media, performance testing (sterility and suitability to support growth of *B. subtilis*) must be performed a minimum of one time, preferably on the first batch prepared per lot.

Suppliers (vendors) of testing materials and components with specific requirements such as sodium hypochlorite and inoculated carriers will be requested to provide verification of the desired specifications. The information and data will be maintained and archived in the project file.

Upon completion of each study, a peer review of the data entry/tabulation will be performed by laboratory personnel. A draft report of the findings or data summary will be compiled and forwarded to each lab's Quality Assurance personnel for review. The designated QAO at each facility will review and comment on the data and supporting information before submission to the statistician. Critical findings will be immediately communicated to the Study Director. Data may be rejected if microbial contamination occurs at a level unacceptable to the Study Director.

Section 6. The Reference Method

Note: Laboratories will only conduct the efficacy component (see C. g)

AOAC Official Method 966.04; Sporicidal Activity of Disinfectants Modified Method

First Action 2006

Applicable to testing sporicidal activity of liquid disinfectants using modified method **966.04** against *Bacillus subtilis* on a hard surface (porcelain carrier). Performance criteria for product efficacy are not impacted. This method has been validated for products containing sodium hypochlorite, peracetic acid/hydrogen peroxide, and glutaraldehyde. See results of the collaborative study supporting the modifications to Method **966.04**.

(1) All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the institutional biosafety regulations. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms refer to the Centers for Disease Control/National Institutes of Health (CDC/NIH) Biosafety in Microbiological and Biomedical Laboratories manual. (2) Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Personal protective clothing or devices are recommended during the handling of these items for purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment may be_employed when appropriate during performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine best course of action. (3) References to water mean reagent-grade water, except where otherwise specified. (4) Commercial dehydrated media made to conform to the specified recipes may be substituted. (5) These microbiological methods are very technique sensitive and technique-oriented, thus exact adherence to the method, good laboratory practices, and quality control (QC) are required for proficiency and validity of the results. (6) Detergents used in washing glassware may leave residues which are bacteriostatic. Test for inhibitory residues on glassware periodically. For procedure, refer to Standard Methods for the Examination of Water and Wastewater, Section 9020, Quality Assurance/Quality Control.

A. Media and Reagents

(a) *Culture Media.*–(1) *Nutrient broth*.–For use in preparing nutrient agar. Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (anatone, peptic hydrolysate of pork tissues, manufactured by American Laboratories, Inc., 4410 S 102nd St., Omaha, NE 68127) to approximately 1 L water. Boil mixture for 20 minutes with constant stirring. Readjust volume to 1 L with water and allow cooling to around 50ºC. Adjust pH to 6.8 ± 0.2 with 1N HCL or 1N NaOH, if necessary. Filter through paper (e.g., Whatman filter paper No. 4). Dispense 10 mL portions into 20 × 150 mm culture tubes or 20 mL portions into 25 × 150 mm culture tubes.

Dehydrated nutrient broth may be substituted – prepare according to the manufacturer's instructions. (2) *Nutrient agar*.–For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized nutrient broth. Boil mixture until agar is dissolved. Adjust pH to 7.2 ± 0.2 if necessary. Dispense 5 mL portions into 16 x 100 mm screw cap tubes. Larger tubes may be used as well. Autoclave for 20 minutes at 121ºC. Remove from autoclave and slant tubes to form agar slopes. (3) *Nutrient agar with 5µg/mL $MnSO_4$:H_20 (amended nutrient agar)*.–For spore production. Suspend 11.5 g nutrient agar in 495 mL water, add 5 mL 500 ppm $MnSO_4$:H_20. Dissolve by boiling. Adjust pH to 6.8 ± 0.2 if necessary. Autoclave for 15 minutes at 121ºC. Pour agar into plates. (4) *Trypticase soy agar*.–Suspend 40 g dehydrated trypticase soy agar in 1 L water and heat gently while stirring. Boil one minute or until completely dissolved. Adjust pH to 7.3 ± 0.2. Autoclave 15 minutes at 121ºC. Pour agar into plates. (5) *Fluid thioglycollate medium (FTM)*.–Suspend 29.5 g of dehydrated fluid thioglycollate medium in 1 L water. Heat to boiling to dissolve completely. Adjust pH to 7.1 ± 0.2 if necessary. Dispense 10 mL portions into 20 ×150 mm culture tubes and autoclave for 15 minutes at 121ºC. Store at room temperature. Protect from light. Note: If after autoclaving the aerated portion of media consumes more than one third of tube, media must be re-boiled by placing tubes in beaker of boiling water. Media can only be re-boiled once. (6) *Fluid thioglycollate medium with 1M NaOH (modified FTM)*.– For subculturing spores exposed to 2.5 M HCl. Suspend 29.5 g of fluid thioglycollate medium in 1 L water. Heat boiling to dissolve completely. Cool and adjust pH to 7.1 ± 0.2 if necessary. Add 20 mL 1M NaOH, mix well. Check final pH and record (pH between 8 and 9 is typical). Dispense 10 mL into 20 × 150 mm culture tubes and autoclave for 15 minutes at 121ºC. Store at room temperature. Protect from light. Note: If after autoclaving the aerated portion of media consumes more than one third of tube, media must be re-boiled by placing tubes in beaker of boiling water. Media can only be re-boiled once. Note: Media can be stored for up to two months.

(b) *Manganese Sulfate Monohydrate*.–500 ppm. Add 0.25 g of manganese sulfate to 500 mL water. Filter sterilize for use.

(c) *Dilute hydrochloric acid*.–2.5M. Use to determine resistance of dried spores. Standardize and adjust to 2.5M as in **936.15**.

(d) *Sterile water*.–Use reagent-grade water. Reagent-grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement. See Standard Methods for the Examination of Water and Wastewater for details on reagent-grade water.

(**e**) *Triton X-100*

(**f**) *Ethanol (40%)*

(**g**) *Test organism.–Bacillus subtilis* (ATCC No. 19659) obtained directly from a commercial supplier (e.g., ATCC).

B. Apparatus

(a) *Carriers.*–Penicylinders, porcelain, 8 ± 1 mm OD, 6 ± 1 mm ID, 10 ± 1 mm length (Available from CeramTec Ceramic, Laurens, SC, www.ceramtec.com, Cat. No. LE15819.)

(b) *Glassware*.– For disinfectant, 25 × 150 mm or 25 × 100 mm culture tubes (Bellco Glass Inc., Vineland, NJ; reusable or disposable 20 × 150 mm (for cultures/subcultures); 16 × 100 mm screw cap tubes for stock cultures. Cap with closures before sterilizing. Sterilize all glassware 2 hr in hot air oven at 180° C or steam sterilize for a minimum of 20 min at 121°C with drying cycle.

(c) *Sterile centrifuge tubes*.–Polypropylene, 15 mL conical tubes with conical bottoms (Corning), from Fisher, or equivalent.

(d) *Water bath/chiller unit.*–Constant temperature for test chemical, capable of maintaining 20 ± 1ºC temperature or specified temperature for conducting the test.

(e) *Petri dishes*.–Plastic (sterile)

(f) *Filter paper*.–Whatman filter paper #2; placed in Petri dishes for storing carriers.

(g) *Test tube racks*.–Any convenient style.

(h) *Inoculating loop*.–Any convenient inoculation/transfer loop for culture transfer.

(i) *Wire hook*.–For carrier transfer. Make 3 mm right angle bend at end of 50 – 75 mm nichrome wire No. 18 B&S gage. Have other end in suitable holder.

(j) *Centrifuge.*–Non-refrigerated (e.g., Eppendorf 5804 R).

(k) *Sonicator.*–Ultrasonic cleaner (e.g., Branson Model 1510).

(l) *Orbital shaker.*–speed range from 25 to 500 rpm (e.g., VWR DS 500).

(m)*Vacuum desiccator*.–For carrier storage. With adequate gauge for measuring 27" (69 cm) of Hg and fresh desiccant.

(n) *Certified biosafety cabinet (Class I or II).*–Recommended for use to maintain aseptic work environment.

(o) *Certified Timer*.–For managing timed activities, any certified timer that can display time in seconds.

C. Operating Technique

(a) *Culture initiation*.– Initiate *B. subtilis* culture (e.g., use nutrient broth to re-hydrate a lyophilized culture, and incubate the broth culture for 24 ± 2 hours at 36 ± 1ºC prior to streak inoculation). Streak inoculate a set (e.g., six) nutrient agar slopes and incubate 24 ± 2 hours at 36 ± 1ºC. Concurrently, perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or use of other identification systems). Following incubation, store at 2-5ºC. Maintain stock culture on nutrient agar slants by monthly (30 ± 2 days) transfers.

(b) *Production of B. subtilis spore suspension*.–Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of nutrient broth and incubate tubes on an orbital shaker for 24 ± 2 hours at approximately 150 rpm at 36 ± 1ºC. Use this culture to inoculate amended nutrient agar plates. Inoculate each plate with 500 µl of broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12-14 days at 36 ± 1ºC. Following incubation, harvest the spores by adding 10 mL cold sterile water to each plate. Using a spreader (e.g. bent glass rod), remove growth from plates and pipet suspensions into 15 mL sterile conical tubes (10 plates = 14 tubes, ~10 mL each). Centrifuge tubes at 5000 rpm for approximately 10 minutes at room temperature. Remove and discard supernatant. Re-suspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5000 rpm for approximately 10 minutes. Remove and discard supernatant. Repeat twice. Re-suspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2-5ºC. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spores. Examine a minimum of five fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores versus vegetative cells should be at least 95%. Spore suspension from multiple plates can be combined and re-aliquoted into tubes for uniformity. Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0×10^{-6} through 1.0×10^{-8}) using pour or spread plating on TSA plates. For pour plating, add molten TSA tempered to 45-55ºC to each plate, swirl, and allow agar to solidify. Incubate plates for 24 ± 2 hours at 36 ± 1ºC and determine titer. Note: When harvested and processed, ten plates of amended nutrient agar should provide 80 - 100 mL of concentrated spore suspension (approx. 10^9 CFU/mL). Diluting the suspension prior to carrier inoculation will be necessary; a titer of 1.0×10^8 to 5.0×10^8 CFU/mL should be adequate to achieve the target carrier count.

(c) *Preparation of porcelain carriers*.–Prior to use, examine porcelain carriers individually and discard those with scratches, nicks, spurs, or discolorations. Rinse unused carriers gently in water three times to remove loose material and drain. Place rinsed carriers into Petri dishes matted with 2 layers of filter paper in groups of 15 carriers per Petri dish. Sterilize 20 minutes at 121ºC. Cool and store at room temperature. Note: Handle porcelain carriers with care when placing in Petri dishes. Minimize carrier movement and avoid excessive contact between carriers that might result in chips and cracks. Wash carriers with Triton X-100 and rinse with water 4 times for reuse.

(d) *Inoculation of Porcelain Carriers*.–Dilute the concentrated spore suspension as necessary with sterile water to achieve carrier counts between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Dispense 10 mL diluted spore suspension into an appropriate number of 25 × 150 mm tubes. Add 10 sterile carriers to each tube containing 10 mL spore suspension, slightly agitate, and let stand 10-15 minutes. Remove each carrier with sterile hook and place upright in sterile Petri dish lined with two sheets of filter paper, no more than 30 carriers per Petri dish. Air dry in biological safety cabinet for approximately 30 ± 2 minutes. Place Petri dishes containing inoculated carriers in vacuum desiccator containing $CaCl_2$ and draw vacuum of 69 cm (27") Hg. Dry carriers under vacuum for 24 ± 2 hours before use in HCl resistance, efficacy testing or carrier counts. Maintain under vacuum for up to three months. Carriers may be used after three months if they meet the acceptable HCl resistance and carrier count criteria. Inoculated carriers should not be used after one year of storage. Sterilize and reuse if necessary (see C.c).

(e) *Spore Enumeration (Carrier Counts).*–Prior to use, determine the carrier counts for each preparation of carriers. Assay 5 randomly selected carriers per preparation. Place each inoculated carrier into a 50 mL plastic, polypropylene conical centrifuge tube containing 10 mL of sterile water. Sonicate carriers for 5 minutes ± 30 seconds. Note: For sonication, place tubes into an appropriately sized beaker with tap water to the level of sterile water in the tubes. Place beaker in sonicator (ultrasonic cleaner) so that water level in the beaker is even with water level fill line on sonicator tank. Fill tank with tap water to water level fill line. Suspend beaker in sonicator tank so it does not touch bottom of tank and so all three water levels (inside test tubes, inside beaker, and sonicator tank) are the same. Following sonication, vortex tubes for 2 minutes ± 5 seconds. Dilute spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile water. Dilute spore suspensions out to 1.0×10^{-5} and plate dilutions 1.0×10^{-2} through 1.0×10^{-5}. Plate each dilution in duplicate using pour or surface spread plating with TSA. For pour plating, add molten TSA tempered to 45-55ºC to each plate. Swirl pour plates to distribute spores evenly and allow agar to solidify. Invert plates and incubate for 24-48 hours at 36 ± 1ºC. Count colonies (by hand or with colony counter). Use dilutions yielding between 30 and 300 CFU per plate (target counts) for enumeration; however, record all counts less than 30. Report plates with colony counts over 300 as TNTC (Too Numerous to Count). Average spore counts per carrier should be between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Do not use carriers with counts outside this range.

(f) *HCl resistance*.–Equilibrate water bath to 20 ± 1ºC. Pipet 10 mL of 2.5M HCl into two 25 × 100 mm tubes, place into water bath, and allow to equilibrate. Start timer and rapidly transfer 4 inoculated penicylinders into an acid tube (2.5 M HCl) with flamed hooks or forceps. Do not allow carriers or transfer device to contact inside of wall of acid tube. Transfer individual carriers after 2, 5, 10, and 20 minutes of HCl exposure to a separate tube of modified FTM. Rotate each tube vigorously by hand for approximately 20 seconds and then transfer carrier to a second tube of modified FTM. For viability control, place one unexposed inoculated carrier in a separate tube of modified FTM. For media sterility, use one tube of modified FTM. Incubate all test and control tubes for 21 days at 36 ± 1ºC. Record results as growth (+) or no growth (0) at each time period. Spores should resist HCl for ≥ 2 minutes to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described.

(g) *Efficacy Test.*–Aseptically prepare disinfectant samples as directed. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use 1.0 mL or 1.0 g of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. For a 30-carrier test, place 10 mL product at dilution recommended for use or under investigation into each of six 25 × 150 mm or 25 × 100 mm test tubes, or use appropriate number of tubes assuming 5 test carriers per tube of test chemical. Place tubes in 20 ± 1ºC water bath and let equilibrate to temperature. Using a sterile hook (or forceps), transfer inoculated carriers sequentially at 2 minute intervals in groups of 5 from Petri dish to test tubes containing sporicidal agent. Use a certified timer to monitor time. Flame hook and allow cooling after each transfer. When lowering carriers into test tube, neither carriers nor wire hook may touch sides of tubes. If interior sides are touched, note tube number. Do not count carrier set if any carrier from that group of 5 yields a positive result; testing another set of five carriers is recommended. Carriers must be deposited into test tubes within ± 5 seconds of the prescribed drop time. Return tubes to water bath immediately after adding carriers. After contact period has been achieved, transfer carriers in same sequential timed fashion into primary subculture tubes containing appropriate neutralizer (10 mL in 20 × 150 mm test tubes). Remove the carriers one at a time from the test tube with sterile hook, tap against interior side of tube to remove excess sporicidal agent, and transfer into neutralizer tube (primary tube). All five carriers must be transferred during each 2 minute interval. Flame hook between each carrier transfer. Move remaining carriers into their corresponding neutralizer tubes at appropriate time. Carriers may touch interior sides of neutralizer tube during transfer, but contact should be minimized. After each carrier is deposited, recap neutralizer tube and gently shake to facilitate adequate mixing and efficient neutralization. Within one hour from when last carrier was deposited into primaries, transfer carriers using sterile wire hook to second subculture tube (secondary tube) containing 10 mL of appropriate recovery medium, one carrier per tube. Move carriers in order, but movements do not have to be timed. Gently shake entire rack of secondary tubes after all carriers have been transferred. Incubate primary (neutralizer) and secondary subculture tubes for 21 days at 36 ± 1ºC. Report results as growth (+) or no growth (0). A positive result is one in which medium appears turbid. A negative result is one in which medium appears clear. Shake each tube prior to recording results to determine presence or absence of growth/turbidity. Primary and secondary subculture tubes for each carrier represent a "carrier set". A positive result in either primary or secondary subculture tube is considered a positive result for the carrier set.

Media sterility controls and system controls (check for aseptic technique during carrier transfer process) are recommended. For media controls, incubate 1-3 unopened subculture medium tubes with the test sample tubes for 21 days at 36 ± 1ºC. For system controls, use sterile forceps or needle hooks to transfer 3 sterile carriers into a tube of test chemical. Transfer system control carriers to neutralizer medium as follows: at start of sample test (prior to first tube), transfer 1 sterile carrier to tube of neutralizer medium. After one half of test carriers have been transferred to neutralizer tubes, transfer a second sterile carrier to tube of neutralizer medium. After all test carriers (last tube) have been transferred to neutralizer tubes, transfer third sterile carrier to tube of neutralizer medium. Transfer system control carriers to secondary subculture medium as follows: immediately prior to initiating transfer of test carriers into secondary subculture medium tubes, transfer first system control sterile carrier from neutralizer medium to tube of subculture medium. After one half of test carriers have been transferred to secondary

subculture medium tubes, transfer second system control sterile carrier to tube of subculture medium. After all test carriers have been transferred to secondary subculture medium tubes, transfer third system control sterile carrier to tube of subculture medium. For each test, include a positive carrier control by placing one inoculated carrier into tube of secondary subculture medium. Incubate controls and test sample tubes together for 21 days at 36 ± 1ºC.

Perform identification confirmation on a minimum of three positive carrier sets per test, if available, using Gram stain and/or plating on TSA. Additional confirmation may be performed using VITEK, API analysis or comparable method. If fewer than three positive carrier sets, confirm growth from each positive carrier set. If both tubes are positive in carrier set, select only one tube for confirmatory testing. For test with 20 or more positive carrier sets, confirm at least 20% by Gram stain. If Gram stains are performed from growth taken directly from positive tubes, the staining should be performed within 5-7 days of conducting the efficacy test.

(h) *Neutralization Confirmation Procedure.*– A neutralization confirmation test must be performed in advance or in conjunction with efficacy testing. This assay is designed to simulate the conditions (i.e., neutralizer, subculture medium, contact time, diluent, concentration of test substance) of the efficacy test and to demonstrate the recovery of a low level of spores (e.g., 5 – 100). Diluted inoculum (e.g., spores of *B. subtilis*) is added directly to the various sets of subculture media tubes (see Table 1). This assay provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer – disinfectant interactions.

Produce a spore preparation according to the procedure for amended nutrient agar. Harvest growth from plates (e.g., five plates) per the method, except re-suspend pellet after final centrifugation step in approximately 100 ml aqueous (40%) ethanol. Determine spore count by serial dilution and plating on TSA. Desirable target of the initial working suspension is 1.0×10^8 to 1.0×10^9 CFU/mL. The suspension may require adjustment to reach target titer. Prepare serial ten-fold dilutions of the inoculum in sterile water out to 10^{-8}. Use 10^{-6}, 10^{-7} and 10^{-8} dilutions to inoculate the neutralizer and subculture media tubes – the target number of spores to be delivered per tube in this assay is 5–100 per tube. Determine spore titer by plating (spread plate or pour plate) each of three dilutions in duplicate on TSA agar. Incubate plates inverted for 24-48 hours at 36 ± 1ºC. Count colonies (by hand or with colony counter). Report plates with colony counts over 300 as TNTC (Too Numerous to Count). Note: A standardized spore preparation adjusted to deliver 5–100 spores/mL may be substituted for the three dilutions of spore inoculum. In addition, spores sheared from inoculated carriers may be used as a working suspension.

Use 5 sterile porcelain carriers (only 3 to be used in the assay). Within 5 seconds, place a set of 5 carriers into a test tube (25 × 150 mm or 25 × 100 mm) containing test chemical; transfer carriers according to section (g). Allow carriers to remain in test chemical per the specified contact time and temperature. After the contact time is complete, aseptically transfer three of the five carriers individually into tubes containing the neutralizer per section (g). This set of tubes is

the Neutralizer/Primary Subculture treatment. Following the transfer of the last carrier into neutralizer tube, transfer each carrier, in sequence, into tube containing secondary subculture medium. This portion of assay is not timed, but should be made as soon as possible. This set is the Secondary Subculture treatment. Following carrier transfer, inoculate each tube (Neutralizer/Primary and Secondary Subculture treatment tubes) with one mL of each of three inoculum dilutions (10^{-6}, 10^{-7} and 10^{-8}). For controls, use three fresh unexposed tubes of neutralizer and three tubes of the secondary subculture medium; also inoculate each control tube with one mL of each of three inoculum dilutions. Include one uninoculated tube of neutralizer and secondary subculture media to serve as sterility controls. See Table 1 for tube inoculation scheme. Incubate all tubes 5-7 days at 36 ± 1ºC. Record results as growth (+) or no growth (0). Note: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

Table 1. Neutralization confirmation procedure – inoculating treatment and control tubes with diluted spore suspension*

Neutralizer-Primary Subculture Treatment	Secondary Subculture Treatment (with Carrier)	Neutralizer-Primary Inoculated Control	Secondary Subculture Inoculated Control
1 mL of 10^{-6} □ Tube 1	1 mL of 10^{-6} □ Tube 1	1 mL of 10^{-6} □ Tube 1	1 mL of 10^{-6} □ Tube 1
1 mL of 10^{-7} □ Tube 2	1 mL of 10^{-7} □ Tube 2	1 mL of 10^{-7} □ Tube 2	1 mL of 10^{-7} □ Tube 2
1 mL of 10^{-8} □ Tube 3	1 mL of 10^{-8} □ Tube 3	1 mL of 10^{-8} □ Tube 3	1 mL of 10^{-8} □ Tube3

*1.0×10^{-6} through 1.0×10^{-8} based on an approx. starting suspension of 10^{8} spores/mL

Confirm a minimum of one positive per treatment and control (if available) using Gram staining and colony morphology on TSA. For each treatment and control group, conduct confirmation testing on growth from tube with fewest spores delivered. *B. subtilis* is a Gram positive rod and colonies on TSA are opaque, rough, dull, round, with irregular margins, and low convex. Colonial variation may be observed and is typical for this strain. Growth in the inoculated controls verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. Note: There may be cases when the neutralizer is significantly different from the secondary subculture media; in these cases, growth may not be comparable. The uninoculated control tubes are used to determine sterility, and must show no growth for the test to be valid.

The occurrence of growth in the Neutralizer/Primary Subculture and Secondary Subculture treatment tubes is used to assess the effectiveness of the neutralizer. No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For a neutralizer to be deemed effective, growth must occur in the Secondary Subculture treatment tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL). Growth in the Secondary Subculture inoculated Control verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance. Growth in the Neutralizer-

Primary inoculated Control should be comparable to the Secondary Subculture inoculated Control if the neutralizer is the same as the secondary subculture media. There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control. The Neutralizer-Primary and Secondary Subculture uninoculated Control tubes are used to determine sterility, and must show no growth for the test to be valid.

Note: For product registration, the U.S. EPA requires the following to demonstrate sporicidal/sterilant-level efficacy: Using AOAC method **966.04**, sixty carriers representing each of two types of surfaces (porcelain penicylinders and silk suture loops) must be tested separately against spores of both *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 3584) on three samples representing three different batches of product, one of which must be at least 60 days old (2 carrier types × 2 test microorganisms × 60 carriers/type = 240 carriers per batch sample; 3 product batches × 240 carriers/batch = total of 720 carriers). The product must kill all of the test spores on all of the 720 carriers without any failures.

References

ASTM International Method E 1054 – Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents

Standard Methods for the Examination of Water and Wastewater. 21st Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC

Biosafety in Microbiological and Biomedical Laboratories. 4th Ed. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health

Section 7. Quantitative Three Step Method

Note: Laboratories will conduct the method in its entirety.

Determining the Efficacy of Liquid Sporicides and Sterilants Against Spores of *Bacillus subtilis* on a Hard Surface Using the Quantitative Three Step Method

The Quantitative Three Step Method (TSM) is suitable for determining the sporicidal activity of liquid sporicidal agents against the genus *Bacillus* on a hard surface. For the purpose of this protocol, the terms sporicide and sterilant are considered synonymous. The TSM is an adaptation of a quantitative micro-method as reported by Sagripanti and Bonifacino (1). See results of the pre-collaborative study supporting the use of the TSM (2). A similar version of the TSM was recently accepted and published by ASTM International (3).

Note: All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the institutional biosafety regulations. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms refer to the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories manual (4). Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Personal protective clothing or devices are recommended during the handling of these items for purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment may be employed when appropriate during performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine best course of action. References to water mean reagent-grade water, except where otherwise specified (5). The methods are technique sensitive and technique-oriented, thus exact adherence to the method, good laboratory practices, and quality control (QC) are required for proficiency and validity of the results.

A. Media and Reagents

(a) *Media.*–(1) *Nutrient broth (NB).*–Dehydrated NB. For use in re-hydrating test organism and preparing nutrient agar. (2) *Nutrient agar (NA).*–For stock cultures slants and plating. Add 1.5% (w/v) Bacto-agar to un-sterilized nutrient broth. Boil mixture until agar is dissolved. If necessary, adjust pH to 7.2 ± 0.2. Dispense 5 mL portions into 16 × 100 mm screw cap tubes. Larger tubes may be used as well. Autoclave for 20 min at 121ºC. Remove from autoclave and slant tubes to form agar slopes. Dehydrated nutrient agar may be substituted – suspend 23 g nutrient agar per L water, dissolve by boiling. If necessary, adjust pH to 6.8 ± 0.2. Autoclave for 15 min at 121ºC. (3) *Nutrient agar with 5µg/mL $MnSO_4$:H_20 (amended nutrient agar).*–For spore production. Suspend 11.5 g nutrient agar in 495 mL water, add 5 mL 500 ppm $MnSO_4$:H_20. Dissolve by boiling. If necessary, adjust pH to 6.8 ± 0.2. Autoclave for 15 min at 121ºC. Pour agar into plates. (4) *Trypticase soy agar (TSA).*–Poured in plates for microbe isolation and spread

plating. (5) *Luria-Bertani broth (LB broth)*.-Dehydrated LB broth (e.g., Difco); suspend 25 g LB broth in 1 L water, mix well, if necessary adjust pH to 7.0 ± 0.2, dispense in bottles and autoclave for 15 min at 121°C; use as neutralizer. (6) *Modified Luria-Bertani broth.*-neutralizer in HCl resistance test, add 20 mL 1M NaOH to 1 L LB broth, mix well, dispense in bottles and autoclave for 15 min at 121°C.

(b) *Manganese sulfate monohydrate*.–500 ppm. Add 0.25 g of manganese sulfate to 500 mL water. Filter sterilize for use.

(c) *Sterile water.*–Use sterile reagent-grade water. Reagent-grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement. See Standard Methods for the Examination of Water and Wastewater for details on reagent-grade water (5).

(d) *Test organism.-Bacillus subtilis* (ATCC No. 19659) obtained directly from a commercial supplier (e.g., ATCC).

B. Apparatus

(a) *Certified biosafety cabinet (Class I or II).*–Recommended to maintain an aseptic work environment.

(b) *Glass coupon*.-Hard surface carrier, 5 × 5 × 1 mm, Erie Scientific Company, Portsmouth, NH; custom order part number EPA-1101 (minimum order of 1000 pieces), single use.

(c) *Sterile 1.5 mL microcentrifuge tubes*.-For exposing carrier to disinfectant, Fisherbrand cat. #05-408-129.

(d) *Sterile centrifuge tubes*.–For preparation of spore suspension, polypropylene, 15 mL conical tubes with conical bottoms, Fisher, cat. #05-538-53D.

(e) *Dissecting forceps*.-For the transfer of carriers, sterile, VWR cat. #25607-195 or Fisher cat. #13-812-42.

(f) *Micropipette*.-Used to make serial dilutions, calibrated.

(g) *Positive displacement pipette*.-For carrier inoculation.

(h) *Desiccator*.–For carrier storage.

(i) *Water bath/chiller unit*.-Constant temperature for test chemical and controls, capable of maintaining 20 ± 1ºC temperature or specified temperature; e.g., Neslab RTE-221 or Nalgene Labtop Cooler.

(j) *Orbital shaker.*

(k) *Microcentrifuge.*

(l) *Microcentrifuge tube lid openers*.-USA Scientific #1400-1508.

(m) *Sonicator*.-Ultrasonic cleaner (Branson Model 1510 Bath Sonicator, or equivalent).

(**n**) *Floating microcentrifuge tube holder*.-For sonication, VWR: #60986-099.

(o) *Hematology rotator*.-For *fraction **C*** recovery**,** Hematology Chemistry Mixer 346–Fisher Scientific; or a suitable mixer/shaker to provide gentle agitation during incubation.

(p) *Vortex mixer.* (also an option for fraction **C** recovery using a vortex adapter).

(q) *Vortex adapters*.-Fisher Scientific cat. #1281161 and 1281211.

(r) *Certified timer*.-For managing timed activities, any certified timer that can display time in seconds.

(s) *Test tubes*.-For sterilizing carriers, 25 × 150 mm.

(t) *95% ethyl alcohol*.- For cleaning carriers.

C. Operating Technique

(a) *Culture initiation*.–Initiate *B. subtilis* culture (e.g., use nutrient broth to rehydrate a lyophilized culture, and incubate the broth culture for 24 ± 2 hours at 36 ± 1ºC prior to streak inoculation). Streak inoculate a set (e.g., six) nutrient agar slopes and incubate 24 ± 2 hours at 36 ± 1ºC. Perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or use of other identification systems). Following incubation, store at 2-5ºC. Maintain stock culture on nutrient agar slants by monthly (30 ± 2 days) transfers.

(b) *Production of B. subtilis spore suspension*.–Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of nutrient broth and incubate tubes 24 ± 2 hr on an orbital shaker at approximately 150 rpm at 36 ± 1ºC. Use this culture to inoculate amended nutrient agar plates. Inoculate each plate with 500 µl of broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device.

Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12-14 days at 36 ± 1ºC. Following incubation, harvest the spores by adding 10 mL cold sterile water to each plate. Using a spreader (e.g., bent glass rod), remove growth from plates and pipet suspensions into 15 mL sterile conical tubes (10 plates = 14 tubes, ~10 mL each). Centrifuge tubes at 5000 rpm for approximately 10 min at room temperature. Remove and discard supernatant. Resuspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5000 rpm for 10 ± 1 min. Remove and discard supernatant. Repeat twice. Resuspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2-5ºC. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spores. Examine a minimum of 5 fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores versus vegetative cells should be at least 95%. Spore suspension harvested from multiple plates can be combined and re-aliquoted into tubes for uniformity. Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0×10^{-6} through 1.0×10^{-8}) on TSA or NA. Incubate plates for 24 ± 2 hr at 36 ± 1ºC and determine titer. Note: When harvested and processed, 10 plates of amended nutrient agar should provide 80 - 100 mL of concentrated spore suspension. Diluting the suspension prior to carrier inoculation will be necessary; a spore titer of approx. 1.0×10^{9} CFU/mL in the suspension should be adequate to achieve the target carrier count.

(c) *Carrier Preparation.*-Visually screen glass coupons (carriers) for scratches, chips, or cracks. Discard those which are damaged or defective. Rinse carriers once with water, rinse 3 times with 95% ethyl alcohol, and finally rinse 3 times with water. Allow carriers to dry. Place in glass tubes (25 × 150 mm) 40 carriers per tube. Steam sterilize 45 min at 121ºC with a 30 min dry cycle or sterilize for 2 hr in hot air oven at 180° C. Cool. Transfer carriers to sterile plastic Petri dishes for inoculation (approx. 40 carriers per dish).

(d) *Carrier Inoculation*.-Transfer 10 µL of spore suspension with a micropipette using aerosol barrier tips or positive displacement pipette onto a 5 × 5 × 1 mm sterile, dry glass coupon. Apply to one central spot on each carrier. Inoculate the necessary number of carries to complete the validation study (approx. 140). Allow carriers to dry for minimum of 1 hour in open Petri dish in a biosafety cabinet, then for a minimum of 12 ± 2 hr in a desiccator. Store inoculated carriers under desiccation for up to 30 days. Glass carriers must be discarded after use. Note: During carrier inoculation, vortex mix inoculum frequently to ensure uniform distribution of spores. Recommend verification of carrier counts (per the method for control carriers) prior to test day; mean counts must be 5.0×10^{6} to 5.0×10^{7} spores/carrier.

(e) *Disinfectant Sample Preparation*.-Aseptically prepare disinfectant samples as directed. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use 1.0 mL or 1.0 g of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Place approximately 1.5 mL of each disinfectant or control (sterile water) in microcentrifuge tubes. Allow to equilibrate to appropriate temperature for 15-30 min.

(f) *Test Procedure Overview*.-A minimum of 3 carriers per disinfectant and 3 carriers for

the water control (control carriers) are required per product test. Three carriers per treatment will be required for the validation study. Use 1 pair of sterile forceps per fraction for each disinfectant. Fractions may be refrigerated briefly to allow for processing of other fractions. If possible, it is recommended that two analysts perform this method so that dilution and plating of the multiple fractions may be conducted as soon as possible. See Appendices TSM-1, TSM-2 and TSM-3 for additional guidance. Note: It is recommended that no more than 3 disinfectant treatments (9 test carriers) plus the water control (3 control carriers) should be tested during the same test period.

Using sterile forceps, carefully transfer 1 inoculated carrier into each microcentrifuge tube labeled *fraction **A***. Avoid touching inoculated area of carrier and sides of microcentrifuge tube. Discard carrier and tube if carrier touches sides of tube. Place *fraction **A*** tubes containing carriers and tubes containing disinfectant(s) and sterile water (control) into chiller water bath at 20 ± 1ºC, or use a labtop cooler to maintain temperature of the tubes. Equilibrate approximately 10 min. Add 400 µL disinfectant (test carriers) or 400 µL sterile water (control carriers) at 15 or 30 ± 5 sec intervals to appropriate microcentrifuge tube (in triplicate). Allow contact of the carriers to the disinfectant or water in *fraction **A*** tubes for the appropriate exposure period.

Following the exposure period, add 600 µL of appropriate ice-cold neutralizer (e.g., LB broth) to each disinfectant *fraction **A*** tube. Add 600 µL LB broth as neutralizer for water control *fraction **A*** tubes. Slightly agitate tubes to thoroughly mix liquid components. Transfer each carrier using 1 pair sterile forceps per carrier set (i.e., 3 carriers) from *fraction **A*** tube to corresponding *fraction **B*** tube. *Fraction B* tubes contain 400 µL ice-cold (0-5ºC) sterile water.

Place *fraction **A*** tubes in microcentrifuge, centrifuge for 6 min ± 30 sec at 13,000 rpm (15,500 × g). Remove 900 µL from each tube without disturbing pellet. Discard supernatant. Carefully add 900 µL ice-cold LB broth to each tube. Repeat 2 additional times. After third centrifugation, remove 900 µL from each tube. Carefully add 100 µL ice-cold LB Broth to each *fraction **A*** tube and resuspend pellet by vortex mixing 5 min ± 30 sec (use the vortex adapter) at midrange speed. Add 800 µL ice-cold LB Broth to each *fraction **A*** tube. Proceed to dilution and plating if another analyst is available, or store *fraction **A*** tubes in refrigerator. Note: Fluid remaining in the *fraction* **A** tubes contains spores dislodged from carrier by exposure to disinfectant or water control. Consistent orientation of the microcentrifuge tubes in the microcentrifuge is important in locating the pellet. The pellet may range in size and be difficult to visualize depending on the treatment. *Fraction **B*** and *fraction **C*** tubes can be evaluated while *fraction **A*** tubes are being centrifuged**.**

Sonicate *fraction **B*** tubes 5 min ± 30 sec using a floating microcentrifuge tube holder placed inside an ultrasonic cleaner. After sonication is complete, add 600 µL ice-cold LB Broth to *fraction **B*** tubes. Vortex approx. 1 minute. Transfer each carrier using 1 pair sterile forceps per carrier set from *fraction **B*** tube to corresponding *fraction **C*** tube (*fraction C* tubes contain 400 µL ice-cold LB broth). Proceed to dilution and plating if another analyst is available, or store *fraction **B*** tubes in refrigerator; however, storage should be limited to 2 hrs. Note: Fluid remaining in the *fraction **B*** tubes contains spores dislodged from the carrier by sonication.

Place *fraction **C*** tubes in a hematology rotator inside incubator for 30 ± 2 min at 36 ± 1ºC. Remove *fraction **C*** tubes after 30 ± 2 min rotation/incubation from incubator. Add 600 µL ice-cold LB Broth to each tube. The carriers remain in the *fraction **C*** tubes. Proceed to dilution and plating if another analyst is available, or store *fraction **C*** tubes in refrigerator. Note: Fluid remaining in fraction C tubes contains spores dislodged from the carrier by gentle agitation for 30 min.

Vortex mix each microcentrifuge tube thoroughly prior to making dilutions. For each fraction and control tube, remove 100 µL and serially dilute 10-fold in 900 µL ice-cold LB broth. For each carrier, direct plate 100 µL of the sufficient dilutions onto TSA or NA to ensure obtaining counts within the target range of 30-300 CFU/plate. Incubate plates a minimum of 24 ± 2 hr at 36 ± 1ºC. Record control counts at 24 ± 2 hr. Record treated carrier counts at 24 ± 2 hr and at 48 ± 2 hr. Confirm the identity of a minimum of one representative colony taken from at least one plate per treatment level (if available) using Gram staining, general growth media (e.g., TSA or NA) or other confirmation procedure. *B. subtilis* is a large Gram positive rod. On general growth media *B. subtilis* colonies are opaque, rough, round low convex colonies with irregular margins. Notes: After plating, dilution tubes may be stored at 2-5 ºC until the results are recorded; the tubes may be used for additional plating if initial plate counts are beyond the recommended target range.

Use counts which fall within 0-300 CFU/plate for calculations. Obtain the total number of spores per fraction by dividing the number of colonies counted in each fraction by its dilution, and account for volume plated. Obtain the total number of spores per carrier by adding the total number of viable spores per fraction for *fractions A, B,* and *C*. Determine log density (LD) of total number of viable spores per carrier by taking Log_{10} (total number of spores per carrier). Determine log reduction (LR) of test carriers by subtracting log density of test carriers from log density of control carriers. Determine average LD and LR for each disinfectant.

(g) *Neutralization Confirmation (EPA Laboratory only).*-Prepare 12 microcentrifuge tubes. Add 400 µL sterile water to tubes 1-6 and 400 µL of disinfectant to tubes 7-12. Allow tubes to equilibrate approximately 10 min at 20 ± 1ºC (or other specified temperature). Add 600 µL neutralizer in ice-cold Luria-Bertani (LB) broth to tubes 4-6 (neutralizer controls). Add 600 µL neutralizer in ice-cold LB broth to tubes 7-9 (ability of neutralizer to inactivate the disinfectant). Gently mix. Add 10 µL of *B. subtilis* spore suspension (approx. 10^9 spores/mL) to each tube and vortex mix for approximately 15 sec. Incubate tubes for 30 min ± 2 min at 20 ± 1ºC (or temperature specified by disinfectant manufacturer). After incubation, add 600 µL ice-cold LB broth to tubes 1-3 (survival controls). Add 600 µL ice-cold LB Broth to tubes 10-12 (disinfectant controls). Serially dilute each tube (10 µL into 990 µL ice-cold LB broth) to achieve plate counts of 30-300 CFU/plate. Plate 100 µL of each dilution onto NA or TSA. Incubate 24 ± 2 hr at 36 ± 1ºC. Count colonies on each plate. Log densities in tubes 1-3 and 4-6 reflect the spore suspension titer and should be within one log of each other. If log densities between tubes 1-3 and 4-6 are greater than one log, then the neutralizer has a sporicidal effect. If the disinfectant is highly effective, log densities in tubes 10-12 should be approximately 6 logs lower than log densities in tubes 1-6. To be an effective neutralizer, log densities in tubes 7-9 should be within 1 log of the

log densities in tubes 1-6. Note: The lead laboratory, the OPP Microbiology Laboratory, will perform this assay on each of the high treatments prior to the initiation of the study to verify the effectiveness of the chosen neutralizers. For this assay, produce a spore preparation according to the procedure for amended nutrient agar. Harvest growth from plates (e.g., five plates) per the method, except re-suspend pellet after final centrifugation step in approximately 100 ml aqueous (40%) ethanol.

(**h**) *HCl resistance*.– Perform on each preparation of inoculated carriers. Conduct TSM procedure on 2.5 M HCl. Follow procedure as specified in part (**f**) with 2 and 5 min exposure periods with three inoculated carriers per time period. Include three control (sterile water) carriers to determine control carrier counts. Use modified LB broth (addition of NaOH) as the neutralizer instead of LB broth for HCl treatments. Perform test at 20 ± 1ºC. Calculate log reduction. Spores should resist HCl for ≥ 2 min (i.e., based on presence of viable spores after 2 min) to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described. Note: Compared to the water control, anticipate 1-2 log reduction of viable spores at 2 min exposure and 3-5 log reduction following the 5 min exposure.

References Associated with the Three Step Method

(1) Sagripanti, J.L. & Bonifacino, A. (1996) Am. J. Infect. Control **24**, 364 – 371

(2) Tomasino, S.F. & Hamilton, M.A. (2006) Unpublished Report. Comparative Evaluation of Two Quantitative Test Methods for Determining the Efficacy of Liquid Sporicides and Sterilants on a Hard Surface: A Pre-Collaborative Study

(3) Standard Test Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Surfaces. (2005) ASTM Designation E 2414 – 05

(4) Biosafety in Microbiological and Biomedical Laboratories (1999) 4th Ed. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health

(5) Standard Methods for the Examination of Water and Wastewater. 21st Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC

Appendix: TSM-1

Three Step Method Processing Sheet

Analyst(s): _____ Test Date: ___/_____/_____

Test Chemical(s): _____

NOTE: Carriers exposed to the disinfectant(s) and water control will be tested in triplicate. It is recommended that no more than three disinfectants plus the water control be tested during the same test period.

The contents of microcentrifuge tubes prior to processing fractions for 1 disinfectant and control.

Treatment	Tube	Contents
Disinfectant	A1-A3	400 µL disinfectant + 600 µL LB broth w/neutralizer (after exposure period)
	B1-B3	400 µL sterile water + 600 µL LB broth (after sonication)
	C1-C3	400 µL LB broth + 600 µL LB broth (after incubation/rotation)
Control	A4-A6	400 µL sterile water + 600 µL LB broth (after exposure period)
	B4-B6	400 µL sterile water + 600 µL LB broth (after sonication)
	C4-C6	400 µL LB broth + 600 µL LB broth (after incubation/rotation)

Prior to Testing (i.e. day before test); as you proceed, add initials to each step in the space provided.

____ Label *fraction **A**, **B** and **C*** microcentrifuge tubes with fraction letter (e.g. A, B, or C) and carrier number (e.g., 1, 2, 3, etc.).
- Fraction tube examples: A1, A2, A3, B1, B2, B3, C1, C2, C3 etc.

____ In advance of testing, prepare *fraction **B*** and *fraction **C*** microcentrifuge tubes:
- Add 400 µL of ice-cold sterile water to *fraction **B*** tubes.
- Add 400 µL of ice-cold LB broth to *fraction **C*** tubes.
- Store these tubes in a refrigerator (2-5°C) until ready for use.

____ Label dilution microcentrifuge tubes (serial dilution blanks) with fraction letter (e.g. A, B, or C), carrier number (e.g. 1, 2, 3, etc.), and dilution (e.g., 10^{-1}, 10^{-2}, 10^{-3}, etc.).
- Serial dilution blank examples: A1-10^{-1}, A1-10^{-2}, A1-10^{-3}, etc.

____ Prepare serial dilution blanks for **A**, **B**, and **C** fractions for all test carriers.
- Add 900 µL of ice-cold LB broth to each dilution blank for test carriers.
- Store these tubes in a refrigerator (2-5°C) until ready for use.

____ Prepare serial dilution blanks for **A**, **B**, and **C** fractions for all control carriers.
- Add 900 µL of ice-cold LB broth to each dilution blank for control carriers.
- Store these tubes in a refrigerator (2-5°C) until ready for use.

____ Sterilize forceps (two pair of forceps for each disinfectant/control tested plus 3 extra).

____ Prepare a 50 mL conical tube with the appropriate neutralizer (place it on ice on test day).

____ Turn on the recirculating chiller and water bath and allow them to reach 20 ± 1°C or the temperature specified.

____ On test day, prepare the disinfectant(s) and place ~1.5 mL of each disinfectant and water control into a microcentrifuge tube
- Be sure to prepare and use the disinfectant within its specified period.
- If the disinfectant requires a dilution, a minimum of 1 mL of the product must be used.
- Ready-to-use disinfectants are tested as received; no dilution is required.

……

3.1.1.1.1.1.1 Fraction A

____ Using sterile forceps, carefully place one inoculated carrier inside each microcentrifuge tube labeled *fraction **A***.
- Avoid touching the inoculated area of the carrier and the sides of the microcentrifuge tube. If the carrier touches the sides of the microcentrifuge tube, the carrier and tube must be discarded.
- These forceps should not be used for any additional transfers, unless sterilized appropriately.

____ Place the *fraction **A*** tubes containing the carriers and the tubes containing the disinfectant(s) and water control into a chiller water bath equilibrated at 20 ± 1°C.
- Allow the carriers, disinfectant(s), and water control to equilibrate for approx. 10 min.

____ Using a 1000 µL micropipette, add 400 µL of the disinfectant or 400 µL sterile water control at 15 or 30 ± 5 sec intervals to the appropriate microcentrifuge tubes (in triplicate).

____ Expose *fraction **A*** tubes in the chiller water bath for the appropriate exposure period.

____ After exposure is complete add 600 µL of the appropriate ice-cold neutralizer to each disinfectant and water control *fraction **A*** tube.
- Slight agitation (e.g., light tapping) may be necessary to thoroughly mix the liquid components.

____ Using one pair of sterile forceps per set of 3 carriers (e.g., per disinfectant), transfer each carrier from its *fraction **A*** tube to its corresponding *fraction **B*** tube.
- Avoid touching the sides of the tube if possible.
- These forceps should not be used for any additional transfers.

Note: Processing of the *fraction B* and *fraction C* tubes can be completed by a second analyst while the *fraction A* tubes are being centrifuged (e.g., sonication of the *fraction B* tubes, transfer of the carrier to the fraction C tubes, rotation of the *fraction C* tubes.

____ Place the *fraction **A*** tubes into the microcentrifuge for 6 min ± 30 sec at 13,000 rpm (15,500 × g).

- Centrifugation #1

____ After *fraction **A*** tubes complete centrifugation #1, carefully remove 900 µL from each *fraction **A*** tube without disturbing the pellet. Discard the supernatant.

____ Carefully add 900 µL of ice-cold LB broth into each *fraction **A*** tube and place the *fraction **A*** tubes into the microcentrifuge for 6 min ± 30 sec at 13,000 rpm (15,500 × g).

- Centrifugation #2

Note: The final volume in each *fraction A* tube is 1000 µL.

____ After *fraction **A*** tubes complete centrifugation #2, carefully remove 900 µL from each *fraction **A*** tube without disturbing the pellet. Discard the supernatant.

____ Carefully add 900 µL of ice-cold LB broth into each *fraction **A*** tube and place the *fraction **A** tubes* into the microcentrifuge for 6 min at 13,000 rpm (15,500 × g).

- Centrifugation #3

____ After *fraction **A*** tubes complete centrifugation #3, carefully remove 900 µL from each *fraction **A*** tube without disturbing the pellet. Discard the supernatant.

____ Carefully add 100 µL of ice-cold LB broth into each *fraction **A*** tube.

____ Resuspend the pellet in the *fraction **A*** tubes by vortex mixing for 5 min ± 30 sec at a midrange speed.

- The use of a vortex adapter is recommended.

____ Add 800 µL of ice-cold LB broth into each *fraction **A*** tube.

____ Proceed to the **Dilutions and Plating** section.

- Place *fraction **A*** tubes in a refrigerator if dilutions are not made immediately. Processing should proceed as quickly as possible.

.....

Fraction B

____ Sonicate *fraction **B*** tubes for 5 min ± 30 sec using a floating microcentrifuge tube holder.

____ After sonication is complete, add 600 µL of ice-cold LB broth to the *fraction **B*** tubes and vortex for one minute.

____ Using one pair of sterile forceps per set of 3 carriers (e.g., per disinfectant), transfer each carrier from its *fraction **B*** tube to its corresponding *fraction **C*** tube.
- These forceps should not be used for any additional transfers, unless sterilized appropriately.

____ Proceed to the **Dilutions and Plating** section.

- Place *fraction **B*** tubes in a refrigerator if dilutions are not made immediately. Processing should proceed as quickly as possible.

……

Fraction C

____ Place the *fraction **C*** tubes in a hematology rotator inside a 36 ± 1°C incubator for 30 ± 2 min.

____ After the 30 min ± 2 min rotation/incubation, remove the *fraction **C*** tubes from the incubator and add 600 µL of ice-cold LB broth to each tube.
- The carriers can remain in the *fraction **C*** tubes.

____ Proceed to the **Dilutions and Plating** section.
- Place *fraction **C*** tubes in a refrigerator if dilutions are not made immediately. Processing should proceed as quickly as possible.

…….

3.1.1.1.1.1.2 Dilutions and Plating

____ Remove dilution blanks from the refrigerator immediately prior to making dilutions.

____ Vortex mix each microcentrifuge tube thoroughly prior to making any dilutions.

____ Serially dilute *fractions **A**, **B**, and **C*** for test carriers.
- Vortex mix each tube prior to making the next dilution.
- Appropriate dilutions for test carriers will be determined via a screen prior to testing.

____ Serially dilute *fractions **A**, **B**, and **C*** for control carriers.

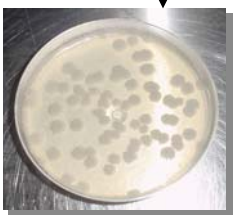
- Vortex mix each tube prior to making the next dilution.
- Appropriate dilutions for control carriers will be determined via a screen prior to testing.

____ Directly plate 100 µL of the dilutions that will yield counts within the target range (30-300) for each carrier.
- Spread using sterile spreader.

____ Incubate all plates at 36 ± 1°C for a minimum of 24 ± 2 hr (see text for details)

Appendix: TSM-2

Three Step Method Schematic

Fraction A	Fraction B	Fraction C
Expose carrier *(x min)*		
Transfer carriers to B tubes		
Centrifuge/wash pellet 3 times	Sonciate carrier 5 min ± 30 sec	
Centrifuge 6 min ± 30 sec each	Vortex approx 1 min	
	Transfer carriers to C tubes	
 pellet	Dilute and plate	Incubate tubes with carrier at 36±1°C in a hematology rotator 30 min. ± 2 min
	Enumerate	
	 Nutrient agar plate with representative *B. subtilis* growth	
Resuspend pellet		
Dilute and plate		Dilute and plate
Enumerate		Enumerate

Appendix: TSM-3

TSM Time Line

Time	Fraction A	Fraction B	Fraction C
2	Exposure (x minutes)		
4			
6	Add 600 µL ice-cold neutralizer		
8	Transfer carriers to B tubes		
10	Centrifugation 1 (6 min ± 30 sec)	Sonication (5 min ± 30 sec)	
12			
14		Add 600 µL ice-cold LB broth	
16	Remove 900 µL supernatant	Vortex (approx. 1 min)	
18	Add 900 µL ice-cold LB broth	Transfer carriers to C tubes	
20	Centrifugation 2 (6 min ± 30 sec)	Dilutions and plating	Incubate tubes at 36±1°C in a hematology rotator (30 ± 2 min)
22			
24			
26	Remove 900 µL supernatant		
28	Add 900 µL ice-cold LB broth		
30	Centrifugation 3 (6 min ± 30 sec)		
32			
34			
36	Remove 900 µL supernatant		
38	Add 100 µL ice-cold LB broth		
40	Vortex (5 min ± 30 sec)		
42			
44	Add 800 µL ice-cold LB broth		
46	Dilutions and plating		
48			
50			Add 600 µL ice-cold LB broth
52			Dilutions and plating

*Time in minutes

Section 8. REPORTING RAW DATA

Information and raw data will be recorded on the test forms and data sheets provided by the Study Director (Appendices B and C). Electronic spreadsheets, provided by the Study Director, will be populated with the data from the hard copy data sheets, peer-reviewed for accuracy, and forwarded to the statistician for analysis. The preparation of media and reagents will be recorded on Media Preparation Sheets provided by the Study Director (Appendix D).

Section 9. STATISTICS AND ANALYZING RAW DATA

For statistical analysis, the Study Director will utilize the services of Dr. Martin Hamilton at the Center for Biofilm Engineering, Montana State University – Bozeman. The statistical analysis will produce estimates of the repeatability standard deviation, denoted by S_r, and the reproducibility standard deviation, denoted by S_R, for each treatment (disinfectant × efficacy level combination) and for each quantitative response (log reduction value and the control carrier log spores per carrier).

In studies such as this, it is not unusual for the dilution series to miss occasionally the counting range of dilutions, thereby providing anomalous counts, either all zeros or all "too numerous to count" (TNTC). For such anomalous data, artificial counts will be substituted. If all dilutions produce TNTC, 300 will be substituted at the last dilution for the fraction (A, B, or C). If all dilutions produce zeros, 0.5 will be substituted at the first dilution.

Overview

The statistical analyses will provide the following information. More detailed descriptions of the statistical methods are presented in the next section.

X **Raw data plots** – the individual data points will be plotted for visual inspection to see trends and effects and to detect outliers or influential observations.

X **Analysis of control carrier spore titers** – the log transformed spores per control carrier will be analyzed using an analysis of variance (ANOVA) two factor, nested, random effects model (details below). These results will describe the "normal range" of control carrier titers for each test method as well as estimates of the S_r and the S_R and the within-test, intra-laboratory, and inter-laboratory sources of variability.

X **log reduction (LR) value** – *LR is the primary quantitative response and most of the statistical work will focus on the LR data.* The LR value will be calculated using the formulas appropriate for each laboratory test (details below). For each combination of test method and test treatment, a one factor, random effects model ANOVA will be conducted to estimate the S_r, the S_R, and the intra-laboratory versus inter-laboratory sources of variability.

X **Mean LR** – for each chemical × efficacy level combination, the mean LR will be calculated along with the associated standard error and confidence interval. For each chemical treatment, a statistical trend test will determine whether the mean LR values increase significantly with increasing efficacy level.

X **Diagnostic plots and tests** – performed routinely to check whether the observations conform to the mathematical assumptions underlying the ANOVA calculations.

X **Presentation of results** – tables and figures will be created to present the results.

- **LR for the AOAC test** – To calculate an LR value that is consistent with each AOAC test result, the P/N formula will be applied (1, 4). Those LR values will be used to calculate S_R for the AOAC test.

- **Percent of the Total Counts by Fraction –** An assessment of the percentage of the total spore counts for each fraction of the TSM will be calculated – the contribution of fraction C will be assessed for importance.

Statistical analysis details

- **Analysis of control carrier spore titers** – The ANOVA will be based on a nested, two-factor, random effects model, similar to the models used in (5, 6). For each combination of test method, the response variable is the log spores per control carrier, the main effect is laboratory (a random effect), the nested effect is replication (a random effect) and the "chance error" is due to the variation among the carriers within the replication. The laboratories taking part in this collaborative study are assumed to be statistically representative of all laboratories that will be conducting these types of sporicide tests in the future. Because the ten participating laboratories were not in fact randomly selected from a population of testing laboratories, the assumption is inaccurate; nevertheless, it is required for the conventional approach to analyzing collaborative studies.

  In standard statistical notation [see for example (7)], let Y_{ijk} denote the log spores for the k^{th} carrier in the j^{th} trial (replication) in the i^{th} laboratory, k=1,2, or 3, j=1,2, or 3 and i = 1,2, …, 10. Then the model is $Y_{ij} = : + L_i + T_{j(i)} + \gamma_{ijk}$, where : denotes the true mean log spores per carrier for that test method and treatment, L_i denotes the effect of the i^{th} laboratory, $T_{j(i)}$ denotes the effect of the j^{th} trial (replication) in laboratory i, and γ_{ijk} denotes the chance error for the k^{th} carrier in the j^{th} replicate test in the i^{th} laboratory. The parameter : is deterministic; it is a specific, but unknown, numerical value. The quantities L_i, $T_{j(i)}$, and γ_{ij} are random variables. According to conventional assumptions, L_i follows a normal probability distribution with mean zero and variance Φ_L^2, $T_{j(i)}$ follows a normal probability distribution with mean zero and variance Φ_T^2, and γ_{ijk} follows a normal probability distribution with mean zero and variance Φ^2 . This model implies that Y_{ij} is a random variable following a normal probability distribution with mean : and

variance $\Phi_L^2 + \Phi_T^2 + \Phi^2$. Let Φ_R^2 (= $\Phi_L^2 + \Phi_T^2 + \Phi^2$) denote the total variance of Y_{ijk}. Conventionally, $\Phi_R = /\Phi_R^2$ is called the *reproducibility standard deviation* and $\Phi_r = [\Phi_T^2 + \Phi^2]^{½}$ is called the *repeatability standard deviation* (5, 8). The ANOVA will provide numerical estimates of the parameters :, Φ_L^2, Φ_T^2, Φ^2, Φ_r^2, and Φ_R^2.

- **Log reduction (LR) values** – For the TSM, the LR is the mean of $\log_{10}$ spores per control carrier minus the mean of $\log_{10}$ spores per treated carrier. Formulas for calculating the LR and associated within-test standard error are presented in Zelver *et al.* (9). For AOAC **966.04**, the LR value will be calculated using the P/N formula presented in the report to AOAC by Tomasino and Hamilton (4)**.**

  The ANOVA will be based on a one-way, random effects linear statistical model. For each combination of test method and chemical, the LR is the response variable, the main effect is laboratory (a random effect) and the "chance error" is due to the variation among independent repeats of the test within laboratories. The laboratories taking part in this collaborative study are assumed to be statistically representative of all laboratories that will be conducting these types of sporicide tests in the future.

  In standard statistical notation [see for example (7)], let Y_{ij} denote the LR value for a specific combination of test method and chemical at the j^{th} replication in the i^{th} laboratory, j=1, 2, or 3 and i = 1, 2, or 3. Then the model is $Y_{ij} = : + L_i + \gamma_{ij}$, where : denotes the true mean LR for that test method and chemical, L_i denotes the effect of the i^{th} laboratory, and γ_{ij} denotes the chance error for the j^{th} replicate test in the i^{th} laboratory. The parameter : is deterministic; it is a specific, but unknown, numerical value. The quantities L_i and γ_{ij} are random variables. According to conventional assumptions, L_i follows a normal probability distribution with mean zero and variance Φ_L^2 and γ_{ij} follows a normal probability distribution with mean zero and variance Φ_r^2 . This model implies that Y_{ij} is a random variable following a normal probability distribution with mean : and variance $\Phi_L^2 + \Phi_r^2$. Let Φ_R^2 (= $\Phi_L^2 + \Phi_r^2$) denote the variance of Y_{ij} ; Φ_R^2 is called the "total variance" in ANOVA textbooks. In the context of germicide tests, $\Phi_R = /\Phi_R^2$ is called the *reproducibility standard deviation* and $\Phi_r = /\Phi_r^2$ is called the *repeatability standard deviation* (5, 8). The ANOVA will provide numerical estimates of the parameters :, Φ_L^2, Φ_r^2, and Φ_R^2. If the estimates of the variances differ insignificantly among test chemicals, the data may be combined across chemicals for purposes of running one ANOVA for that test method. If it is appropriate to do so, combining the data will produce more reliable estimates of the parameters. For the TSM, by using the within-test standard error associated with each LR, it will be possible to partition out the within-test component of variance from Φ_r^2.

X Mean LR – The mean for each test chemical and test method combination will be estimated by the ANOVA. The formula for the standard error of the mean depends on the results of the ANOVA and the equations will be derived by the statistician, as in (10). The confidence intervals will be based on normal distribution theory.

It is of interest to determine whether each method is sensitive enough to correctly order

treatments known to have low, medium, and high efficacy. For each method and disinfectant, a trend test will be conducted to determine whether the log spores per treated carrier increases significantly with known efficacy. The trend test will be a test for a significant mean slope based on a simple linear regression model. The efficacy levels will be coded as 1, 2, and 3, going from low to high efficacy.

- S_r and S_R of LR values for the TSM – It is desirable for the standard deviations to be small. For disinfectant tests, the AOAC has no specifications for concluding that a standard deviation is acceptably small. Some guidance is provided by a recent literature review which showed that, for established suspension and dried surface disinfectant tests, S_r ranged from 0.2 to 1.2 with a median of 0.4 and S_R ranged from 0.3 to 1.5 with a median of 0.8 (6). It would be reasonable to claim that the S_r and S_R are acceptably small if they fall within these ranges.

X Diagnostic plots and tests – conventional plots and tests of residuals will be used to check the homogeneous variance and normality assumptions underlying the ANOVA. The Anderson-Darling test will be used to check normality.

X The % of the total spore counts for each fraction of the TSM will be evaluated – the contribution of fraction C will be assessed for importance.

Section 10. REFERENCES ASSOCIATED WITH THE VALIDATION PROTOCOL

1. Tomasino, S.F. & Hamilton, M.A. (2006) Unpublished Report. Comparative Evaluation of Two Quantitative Test Methods for Determining the Efficacy of Liquid Sporicides and Sterilants on a Hard Surface: A Pre-Collaborative Study
2. Sagripanti, J.L. & Bonifacino, A. (1996) *Am. J. Infect. Control* **24**, 364 – 371
3. Standard Test Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Surfaces. (2005) ASTM Designation E 2414 – 05
4. Tomasino, S.F. & Hamilton, M.A. (2006) Unpublished Report. Modifications to the AOAC Sporicidal Activity of Disinfectants Test, Method 966.04: Collaborative Study
5. Youden, W.J. and Steiner, E.H. (1975) Statistical Manual of the AOAC. AOAC: Arlington, VA
6. Tilt, N. and Hamilton, M.A. (1999) *JAOAC Int.,* **82**, 384 – 389
7. Neter *et al.*, (1996) Applied Linear Statistical Models – 4th edition, McGraw-Hill:Boston
8. Helrich, K., editor, (1990) Official Methods of the AOAC, AOAC: Arlington, VA, p. 681, 1990
9. Zelver, N. et al. (2001) Methods in Enzymology - Biofilms II, R.J. Doyle, editor, 337:363 – 376
10. Johnson, K., Lundman, R., and Hamilton, M. (1993) Efficient Sampling Designs for Microbial Processes: A Case Study. *Journal of Microbiological Methods*, **18**:69 – 81

Section 11. APPENDICES

A. EPA Quality Assurance Project Plan

B. AOAC Method Data Sheets

C. TSM Data Sheets

D. Media and Reagent Preparation Sheets

E. Parameters for Testing Chemicals

F. TSM Log Reduction Data

G. Material Safety Data Sheets

H. Safety Checklist

Appendix C – Guidelines for the Development and Validation of Nucleic Acid Amplification (PCR) Based Microbiological Methods

Introduction

This section provides initial guidance that specifically considers how to develop and validate nucleic acid amplification-based methods for environmental sample analyses. This guidance specifically refers to methods incorporating the polymerase chain reaction (PCR) amplification technique; however, this guidance should also be applicable to methods incorporating other nucleic acid amplification techniques.

1.0 The PCR Method: General Background

A PCR method encompasses a series of molecular procedures including 1) sample preparation, 2) nucleic acid amplification with a select PCR assay, 3) visualization of results, and 4) interpretation of data. PCR amplification is an *in vitro* enzymatic technique for rapidly increasing the quantities of specific nucleic acid segments present in small or complex samples to sufficiently high levels to allow their detection by optical, physical and other methods. PCR has been routinely used in clinical and food microbiology for many years. For a detailed review of PCR methods, please see (Sambrook and Russell 2001 McPherson and Moller 2006).

Two common approaches for PCR amplification are end-point and real-time detection. End-point detection is the classical approach where the presence of the target sequence is determined by analysis for the amplified copies by an independent technique (e.g., gel electrophoresis) after the reaction has been completed. This approach is most commonly used as a presence/absence test. Real-time PCR monitors production of target sequence copies throughout the amplification process, either by use of a specific fluorescently-labeled probe sequence or with a nonspecific intercalating dye, and yields both qualitative and quantitative data. Detailed explanations of real-time PCR detection processes are available elsewhere (Sambrook and Russell 2001, Bustin 2004). Both end-point and real-time PCR approaches can easily detect a specific target sequence in a complex mixture of nucleic acids and often with a limit of detection of as little as one copy per reaction, yet the potential for poor DNA purification efficiency and/or potential inhibition of amplification from environmental samples often means that many target microbes are required in the original sample. Nonetheless, various advantages of PCR methods have led many researchers to develop applications of this technology for environmental science.

2.0 Method Selection

When considering a particular PCR method for validation, it is necessary to clearly define the intended use of the method, manner of data analysis and the environmental matrix or matrices of interest. The decision to select a PCR-based analytical method for validation should also take into account the criteria specified in Section 2.2 of the main report. Criteria that may be particularly important in deciding between a PCR or culture-based method for an intended purpose are accuracy, precision, and the relative cost and level of training required for each approach. In general, this justification for PCR methods requires instrumentation, with expected advantages in sensitivity, specificity, rapidity, and throughput; or by the unavailability of a comparable culture method for the target microorganism(s) of interest. Another consideration may be the objective of detecting viable organisms. PCR methods generally will be unable to make this distinction although more recent modifications have shown promise in this regard, e.g., reverse transcription PCR for detection of labile messenger RNA molecules or intermediates of ribosomal RNA processing and pretreatment of samples with intercalating dyes (such as propidium monoazide) that may only permeate cellular membranes of nonviable organisms (Nocker *et al*. 2007).

3.0 Method Development and Optimization

A sound method development and optimization process is critical for a successful validation. The following section briefly describes some of the key elements to consider during the development and optimization of a PCR method.

3.1 Design of Primers and Probes

PCR method development normally begins with the design of PCR assay primers and, in the case of real-time PCR, probes. A primer is a strand of nucleic acid that serves as a starting point for DNA replication. PCR assays typically require two primers to target a specific genomic region. PCR primers are short, chemically synthesized oligonucleotides, with a length of about 20 bases. They are designed to hybridize to a DNA target, which is then copied by a DNA polymerase. Probes are another type of oligonucleotide used in many quantitative real-time PCR applications. Probes are typically designed to anneal within a genomic region amplified by a specific set of PCR primers, which can be used to increase PCR assay specificity, Probes

normally contain both fluorophore and quencher molecules. The quencher molecule quenches the fluorescence emitted by the fluorophore during excitation when in close proximity. This predictable fluorescence trend can then be used to estimate the concentration of target DNA molecules after each step of amplification.

After determining the intended use of a particular PCR method, the first consideration may be the choice of the genomic region that will be targeted by the assay. For example, a PCR method intended to detect and enumerate fecal pollution may target genomic regions that are unique to bacteria associated with fecal contamination. The prediction of specificity and sensitivity of the primers and/or probe for the intended genetic target will typically be the next consideration and is determined from the available database of sequences from both target and non-target organisms. Several publicly (such as GenBank) and commercially available computer software programs are available that can aid in this process.

Concurrent with this process should be the evaluation of the candidate primer and/or probe sequences for their abilities to satisfy a number of basic requirements for PCR amplification. Some of these requirements include primer sequence lengths and melting temperatures, G + C content, secondary structure, and hybridization stringency, as well as other features. Despite the increasing sophistication of PCR assay primer and probe design programs, different primer pairs for the same target sequence region can exhibit significant differences in performance (He *et al* 1994*).* For this reason, there is no substitute for experimental testing of a candidate PCR assay. Part of this process may also include the optimization of conditions under which the assay is performed.

3.2 Optimization of Reaction Conditions

Variable conditions that can be examined include thermal cycling times and temperatures, salt and polymerase co-factor (e.g., magnesium) concentrations, primer and probe concentrations, and nucleic acid quantity in the reaction. These variables may also be characterized within the context of a single PCR instrument and reagent system or with multiple combinations of commercially available instruments and PCR reagents. With so many conditions and their interactions, a factorial design works very well for PCR optimization.

3.3 Sample Preparation

A PCR method consists of not only the target sequence PCR assay, but also the procedures for preparing samples for these analyses. A requisite component in developing a PCR method is therefore the selection and optimization of the sample preparation procedure. At a minimum, this procedure includes the isolation and recovery of nucleic acids across a range of environmental sample quantities determined by the intended use of a particular PCR method. In some cases, this procedure may also include sample concentration and purification of the extracted nucleic acids. The primary variables that must be considered in the selection and optimization of a sample preparation procedure are target sequence recovery efficiency and inhibition of target sequence amplification by co-extracted substances originating from the environmental sample matrix. Hence, controls should be designed to estimate sample preparation efficiency as well as PCR assay inhibition.

3.4 PCR Inhibition Control

Partial or complete inhibition of PCR amplification can be caused by a number of contaminants most often resulting from insufficient purification during sample preparation. There are a number of inhibitory substances that can co-extract with nucleic acids recovered from environmental samples due to similarities in solubility, charge and/or molecule size (Wilson 1997). The resulting PCR inhibition can completely prevent the amplification of target nucleic acids or reduce sensitivity resulting in false negatives or incorrect real-time quantitative PCR measurements. Thus, the inadvertent presence of PCR inhibitors can confound even the best designed and optimized PCR method. The addition of an amplification control sequence to each reaction can be used to distinguish between a false negative, true negative, or PCR inhibition. Amplification controls can be designed to amplify either simultaneously with the target nucleic acid or in a separate reaction. The amplification control signal should always be produced even when there is no target nucleic acid present. There are many strategies to construct, detect, quantify, and store IAC templates. Some design approaches require substantial technical skills, while others rely on more basic techniques. It is important to consider the advantages and drawbacks, as well as the intended use of the PCR method when selecting a particular IAC strategy. For a detailed discussion, see Hoorfar *et al.* 2004. At this stage, most

IAC are not suited to also check for nucleic acid extraction performance, hence additional sample processing controls may also be required.

3.5 Sample Processing Control

Controls that can be used to monitor the efficiency of DNA recovery from the sample are commonly recommended and are often referred to as sample processing controls (SPCs). In addition to their use for estimating sample preparation efficiencies, data resulting from these analyses can also be used to adjust results for variability in sample preparation efficiency in a particular environmental matrix compared to standard laboratory conditions. Depending on the sample preparation method and the target organism involved, an SPC can consist of a nucleic acid target sequence or whole organism that is added to the sample prior to processing. Whole organisms that are as similar as possible to the true target organism(s) in their physical properties are preferable.

3.6 Preparation and Use of Standards for Quantitative Real-time PCR Applications

Estimates of absolute target DNA concentration in environmental samples depends upon the quality of the nucleic acid standards that are employed. For microbial gene targets, DNA standards can consist of purified preparations of genomic DNA, PCR amplicons, or synthetically prepared DNA molecules. Each of these types of standards has advantages and disadvantages. Genomic DNA standards have the advantage of most closely representing the actual template of a particular PCR assay and any possible effects they may have on amplification efficiency. The potential disadvantages of these standards lie in the difficulty that they create for accurately determining the concentration of target sequence copies present and in obtaining reproducible DNA preparations. Estimates of the target gene copy concentrations in these preparations should be verified by limiting dilution PCR analysis or by some other comparable means. Amplicons and synthetic DNA standards can be used in PCR assays for which genomic DNA is not available and are also more amenable to accurate quantitative estimation of target sequence copy concentrations. However, the amplification efficiency of these templates should be compared, when possible, with genomic DNA templates to determine equivalency. For target nucleic acids of major importance, internationally accepted standard reference materials should be established. For example the World Health Organization has established quantitative standards for HCV,

HBV, and HIV viruses. Similar approaches for the establishment of reference standards for other real-time PCR applications should be possible through certification by national or international organizations such as NIST or NIBSC. Analysis results of a reputable commercial or other non-commercial organization may be sufficient for the certification of quantitative reference standards for some real-time PCR applications.

3.7 Real-Time Quantitative PCR Data Analysis

For most real-time quantitative PCR applications, a cycle threshold (CT) measurement is used to estimate the DNA target concentration in a particular environmental sample. Because real-time quantitative PCR is based on the theoretical premise that there is a log-linear relationship between the starting amount of DNA target in the reaction and the CT value that is obtained, the CT value can then be used to estimate the initial concentration of a DNA target from an unknown sample. Two general strategies are often used to make these estimates, including relative and absolute approaches (ABI: Essentials of Real Time PCR. *Applied Biosystems* 2006). A relative quantification approach measures the change in target DNA concentration relative to another reference sample. In contrast, absolute quantification approach is achieved by using a standard curve, constructed by amplifying known amounts of target DNA in a parallel set of reactions (ABI: Absolute Quantitation Using Standard Curve Getting Started Guide**.** *Applied Biosystems* 2006). The approach selected should be clearly described and should adequately address uncertainties associated with a particular PCR method. For example, uncertainty can arise within and between experiments from numerous sources such as inconsistencies in quality of reagents, pipet calibration, as well as dilution preparation and storage of standards. Any of these factors could significantly alter CT measurements from experiment to experiment. Therefore, estimation and propagation of uncertainty throughout data analysis becomes critical to account for sources of variability and make reasonable estimates of environmental sample DNA target concentrations.

4.0 Quality Assurance and Quality Control (QA/QC)

Proper laboratory QA/QC procedures are essential to a successful PCR method. The sensitivity required for the synthesis of billions of target nucleic acid molecules make PCR methods prone to contamination from extraneous DNA, which can lead to false-positive results.

Laboratories performing PCR methods should establish a sufficient number of controls for the detection of contaminating DNA molecules that can be introduced during sample preparation and PCR amplification. Thus, strict protocols must be followed to assure that personnel, facilities, workflow, equipment, disposables, negative controls, and laboratory cleaning practices are adequate to avoid contamination of results. It is also important that instrument QC procedures be followed. Laboratory QA/QC guidance for PCR methods are discussed in "Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples" (USEPA 2004). As part of a lab's QA program the fact that QA procedures have been followed should be clearly documented.

5.0 PCR Method Performance Criteria

Although the general definitions of the performance criteria described in Section 2.4 of the main document remain the same, this section provides examples of approaches for deriving the required performance criteria for methods utilizing PCR. It should be noted that these examples are not all-inclusive and that performance criteria should be measured across the entire method from sample collection through sample preparation to PCR amplification and interpretation of results. In addition, performance criteria that require the use of a standard or control spike may utilize DNA targets reported as cell equivalents, genome equivalents, copies of DNA and/or mass of DNA depending on the intended use of the PCR method. Metrics for report values should be detailed in the method.

5.1 Specificity and Sensitivity

Specificity is the ability of a PCR method to discriminate between target sequences. There are many factors that can impact the specificity of a PCR method such as primer design; degeneracy of the primers; presence of heterologous nucleic acids originating from a sample of interest; total amount of nucleic acids in the PCR experiment; quality of extracted nucleic acids; PCR amplification conditions such as buffer composition, primer concentration, and thermal cycling parameters; matrix effects such as co-extracted impurities that can cause inhibition; non-specific fragment amplification, and quality of reference samples available. It is important to note that specificity values are determined from a collection of reference samples, and therefore this estimate is only as good as the available reference standards. Reference collections such as

DNA sequence databases can be riddled with errors and are routinely updated making it even more challenging to standardize specificity testing. Poor specificity can be identified in numerous ways including amplification of incorrect sized target sequences, background smears in samples that contain fragments of the correct size, unexpected bands in controls that are not attributable to cross-contamination, and correct sized fragments that fail to be confirmed by another technique (e.g. restriction analysis, DNA sequencing, hybridization). However, some PCR methods are designed to detect target sequences in complex mixtures of heterologous nucleic acid preparations that can significantly vary in composition and amount from one sample to the next. For these methods, mixture studies that measure the ability of a given PCR method to obtain reliable results from mixed source samples may be necessary. Mixture ratios should represent the range of conditions that may be encountered when implementing the method (SWGDAM 2004).

Sensitivity is the probability that a PCR method will classify a test sample as positive, given that the test sample is a "known" positive. Sensitivity can be affected by characteristics of the matrix and can be measured under laboratory conditions with a target spike into a characterized matrix such as molecular grade water (laboratory sensitivity) or uncharacterized matrix such as an environmental sample (field sensitivity).

5.2 Precision

For end-point PCR detection methods that generate qualitative data, measures of precision, such as repeatability and reproducibility have little value. Quantitative real-time PCR applications allow for more refined measurements of precision in which the amount of variability observed from a series of repeated measurements of a reference standard can be determined. Precision is often expressed as the relative standard deviation (RSD), which is the absolute value of the percent coefficient of where:

$$RSD = (\text{standard deviation of measurements} \times 100) / \text{mean} \qquad \text{Equation (6)}$$

Precision can be reported for the amplification assay or for the entire method. Figure C-1 illustrates an example of calculating precision for a quantitative real-time PCR assay.

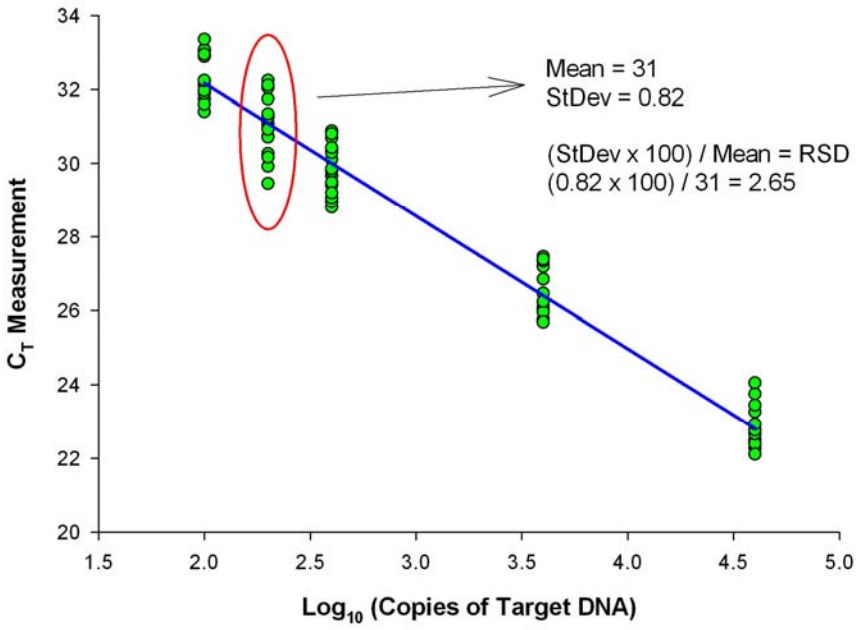


Figure C-1. Example Calculation of precision expressed as RSD for raw C_T data from a fitted curve for quantitative real-time PCR. Data points in circle represent independent measurements of the same DNA standard concentration used to calculate a mean and standard deviation.

For many chemical and culture-based methods, reference standards are typically defined by the International System of Units and are maintained by national or international organizations. However, for most quantitative real-time PCR methods, nationally or internationally recognized reference standards are not available, making it more challenging to establish precision within and between laboratories. To date, there is no commonly accepted practice for determining precision for a quantitative real-time PCR method. This is, in part, due to a lack of standardized reference samples, but also because a quantitative real-time PCR method includes multiple steps such as sample preparation, amplification, and data analysis that can each introduce uncertainty and error. Ideally, a standardized reference sample should be included through the entire quantitative real-time PCR method, resulting in an estimated concentration measurement.

Precision is sometimes classified into repeatability and reproducibility (Section 2.4.2 of the main document). Repeated measurements generated on the same day, with the same lot of reagents, on the same instruments, by the same technician can be used to calculate quantitative

real-time PCR method repeatability. In contrast, repeated measurements generated from the same process among different lots of reagents, instruments, and technicians over longer periods of time can be used to estimate the reproducibility of the method.

5.3 Accuracy and Bias

Accuracy is defined as the ability of a quantitative real-time PCR method to correctly enumerate a "known" number of DNA targets. Accuracy should be measured with blind spikes of DNA targets and blind spikes of the whole organism. Known DNA targets would be spiked into molecular grade water and also a characterized matrix with known quantities of potential chemical and microbial interferences as well as inert substances typical of the intended sample substrate (laboratory accuracy) or uncharacterized matrix such as an environmental sample (field accuracy). Samples of uncharacterized matrices containing blind spike material should be split for analysis in different laboratories. Careful preparation and handling of standards for accuracy measurements is critical for the estimation of PCR method accuracy. Bias is the difference between the observed measurement/estimate of DNA copy number and the known standard concentration.

5.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD is the minimum amount of target sequence that can be detected with a given level of confidence in a well characterized background matrix (e.g., distilled water or a buffer solution). LOQ is similar to LOD except it is a range of the upper and lower bounds that can be quantified with a predetermined acceptance level of precision, accuracy and specificity. Acceptance levels are subjectively determined based on the intended use of a particular method. For example, 40 CFR Part 136 Appendix B contains equations for calculating detection limits for methods promulgated by EPA and defines the detection limit as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. It should be noted that a 99% confidence may not be necessary for all methods. It is important to establish the LOD and/or LOQ for a PCR method to generate a baseline performance level to which a researcher can compare performance in uncharacterized matrices where there could be potential amplification inhibitors. LOD/LOQ measurements are easily determined for both end-point detection and real-time PCR approaches. However, end-

point detection is typically based on visual detection of PCR products and can vary somewhat among analysts, types of electrophoresis gels, and nucleic acid staining agents.

5.5 Sample Limit of Detection (SLOD)

SLOD is the minimum amount of target sequence that can be detected with a given level of confidence in an uncharacterized background matrix (e.g., environmental sample). SLOD estimates are specific to a particular matrix background and can vary from one sample to the next. SLOD experiments are performed in the same manner as LOD experiments (Section 2.4.4), however, reference standards are spiked into the environmental sample matrix rather than laboratory grade water or buffer. SLOD can be expressed as the minimum number of cell equivalents, genome equivalents, target sequence copies, or mass of target sequence with a confidence interval that can be detected (end-point PCR) or enumerated (quantitative real-time PCR).

5.6 Linearity, Range of Quantification (ROQ), and Amplification Efficiency

A standard curve, constructed by testing a series of serial dilutions of known concentrations or copy numbers, provides important information for validating qPCR methods. Due to the stochastic nature of nucleic acid amplification, especially at low nucleic acid target concentrations, five to eight dilutions (e.g., 10-fold) bracketing the range of concentrations (for which the PCR method will be used) are used to characterize the relationship between nucleic acid concentration and response (FDA 2001). From this data a linear plot of C_T vs. the logarithm of the target copy number is generated (Figure C-1). Linearity, expressed as the coefficient of determination R-squared (R^2); (Moore and McCabe 1989), is a measure of the range of target nucleic acid concentrations for which a quantitative PCR test result is directly proportional to the nucleic acid target concentration. ROQ is defined as the range of nucleic acid target concentrations that are detectable with an acceptable level of precision, accuracy, and specificity. Linearity and ROQ are determined by testing different concentrations of standard nucleic acid control samples to generate a plotted curve. For real-time PCR, R^2 is a statistical measure of how well a regression line approximates C_T values obtained from repeated testing of nucleic acid standards (Figure C-2). An R^2 value of 1.0 (100%) indicates a perfect fit. The degree to which the plotted curve conforms to a straight line indicates the PCR method linearity. ROQ can then

be calculated by determining the difference between upper and lower (LOQ) bound concentrations where quantitative measurements are linear and within acceptable levels of precision, accuracy, and specificity.

Amplification efficiency is calculated from the slope of a qPCR standard curve and is expressed as:

$$\text{Amplification Efficiency} = (10^{(1/\text{-slope})})\text{-}1 \qquad \text{Equation (7)}$$

Under ideal conditions, the PCR product doubles after each cycle during exponential growth. This relationship can be numerically expressed as an exponential amplification of 2.0, which is equivalent to amplification efficiency of 100%. Amplification efficiency can be influenced by factors such as length, G/C content, and secondary structure of the amplification product. To assure accurate and reproducible estimates of DNA target concentration, the slope of the standard curve should indicate an amplification efficiency as close to 100% as possible.

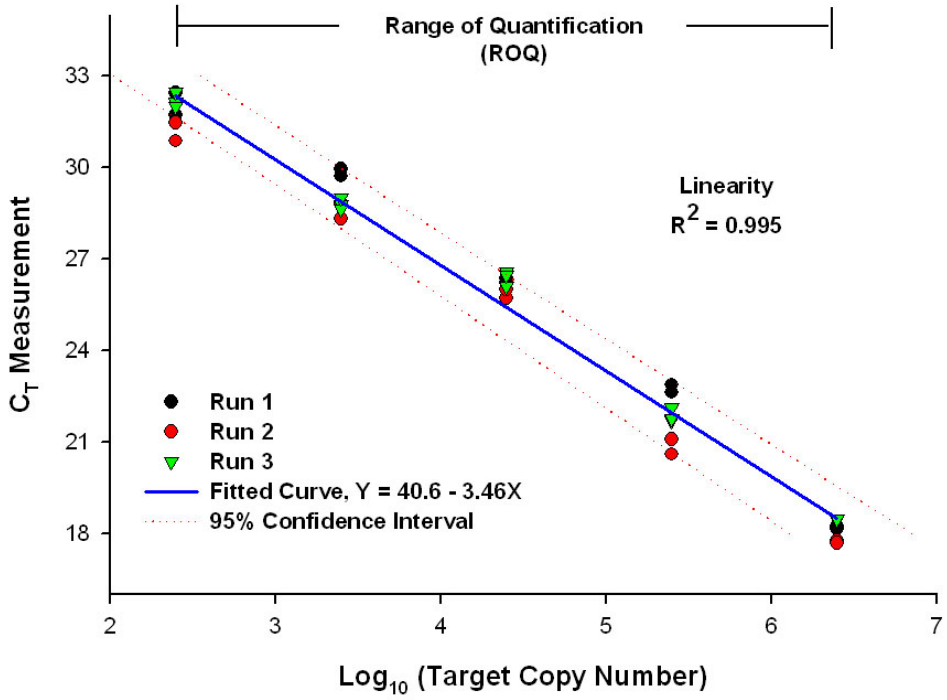


Figure C-2: Example of determining the linearity and ROQ for a quantitative real-time PCR assay. The fitted curve line with a 95% confidence interval represents the best fit line based on C_T measurement from three independent experiments testing a broad range of target nucleic acid standard concentrations. The linearity of the

assay is reported as an R2 value. The ROQ is depicted as the range of nucleic acid standard concentrations detectable within a 95% confidence interval.

5.7 Ruggedness

Ruggedness (unavoidable changes) is the ability for a PCR method to perform within acceptable precision and accuracy performance levels under normal but unavoidable variable conditions; determined by testing identical samples under variable conditions. Factors such as reagent stock stability, analyst to analyst variation, use of different thermal cycling instruments, laboratory to laboratory variation, nucleic acid target stability, and optimal performance over time can all contribute to the ruggedness of a PCR method (FDA 2001). Ruggedness can be expressed as the change in precision. For qualitative end-point PCR methods, ruggedness can be determined by measuring changes in false positives/false negatives and LOD. For real-time quantitative PCR methods, ruggedness can be evaluated using the Horwitz equation (Horwitz 1995) where the relative standard deviation of reproducibility (RSD_R) is given as:

$$RSD_R \text{ (predicted)} = 2^{(1-0.5\log C)}; \text{ and} \qquad \text{Equation (8)}$$

$$\text{HORRAT} = RSD_R \text{ (observed)} / RSD_R \text{ (predicted)} \qquad \text{Equation (9)}$$

Where:

C is the observed concentration expressed as a decimal fraction and log is base 10. The ratio between observed and predicted RSD_R values is designated HORRAT and can be used as an indication of acceptability (HORRAT ratios $\leq$ 1.0 indicates acceptable precision).

6.0 Multilaboratory Validation Studies

A document describing the PCR method should be prepared as described in Section 2.5 of the main document. General guidance for the performance of collaborative validation studies, as described in Section 2.6 of the main document should be applicable for these methods.

6.1 Influence of PCR Platform and Reagent System

It is contrary to general policies within EPA that discourage endorsement or recommendation of specific commercial products; however, it may be necessary to treat assays that utilize different platforms as different methods for the purpose of validation. The popularity

of the PCR technique has given rise to the availability of a wide choice of thermal cycling instrument and amplification reagent systems from different commercial vendors. Since PCR methods are typically developed and optimized with only one such system, the validation of these methods would be limited to that system under ideal circumstances. Particularly with respect to different instruments, however, this practice would limit the number of participants that may be available for multiple laboratory studies, as well as the general acceptance of a method by its anticipated end-user community.

Prior to performing a collaborative validation study involving multiple instrument and/or reagent systems, it is highly desirable to experimentally assess the equivalence of method performance with all systems being considered. Particular attention should be paid to any significant differences in specificity and sensitivity with these systems. Assay specificity may be affected by different amplification reagents (Siefring *et al.* 2008). Preliminary assessments of specificity can be performed through the analysis of common DNA standards from one or more non-target controls that are closely related to the target of interest. If variability in specificity is observed, it may be possible to adjust the annealing temperature of the thermal cycling protocol for each respective reagent and instrument to bring it within acceptable levels. It should be noted, however, that comparable specificity may be difficult to demonstrate for all possible target and non-target organisms.

The sensitivity of an assay on different instruments can be predetermined by the analysis of common DNA standards. Large differences in sensitivity with a given reagent may require instrument-specific adjustments in thermal cycling parameters or the exclusion of less sensitive instruments from being utilized in a validation study with that reagent. Due to differences in optics, some variability in sensitivity may be unavoidable with different instruments but this may not necessarily exclude the use of the less sensitive instruments. Since such differences in sensitivity should apply equally to the analysis results of test samples and DNA standards on each instrument, the comparability of quantitative measurements should not be affected within each instrument's respective ROQ. If acceptable minor differences in sensitivity and LOQ are observed, these characteristics may be defined beforehand for each platform if economically feasible, and the differences taken into consideration in the design of a validation study.

7.0 Potential Differences between PCR Chemistries

Several different probe and non-probe based PCR product detection chemistries are also available for real-time PCR assays (Wittwer and Kusukawa 2004). Moreover, product detection reagents employing these chemistries are often available from numerous commercial vendors. Because of the high potential for the performance of a PCR assay to be altered by these different chemistries, as well as by different commercial sources of the product detection reagents, it is recommended that the chemistry and commercial source of reagents used for method development be specified for each real-time PCR method. Alternative sources of reagents can be designated if their products can be experimentally demonstrated to provide equivalent performance in the method to that of the reagents from the originally specified source.

8.0 References

1. Bustin, S.A. 2004. Quantification of Nucleic Acids by PCR. In S.A. Bustin, A-Z of Quantitative PCR. La Jolla, CA. International University Line.

2. FDA. 2001. Guidance for Industry: Bioanalytical Method Validation. U.S. Food and Drug Administration.

3. He, Q, M. Marjamäki, H. Soini, J. Mertsola, and M.K. Viljanen. 1994. Primers are decisive for sensitivity of PCR. Biotechniques 17: 82, 84, 86-7.

4. Hoorfar, J., B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner, and P. Fach. 2004. Practical Considerations in Design of Internal Amplification Controls for Diagnostic PCR Assays. Journal of Clinical Microbiology 42:1863-1868.

5. Horwitz, W. 1995. Protocol for the design, conduct and interpretation of method performance studies. Pure Appl. Chem. 67:331-343.

6. McPherson, M.J. and S.G. Moller. 2006 PCR (The Basics). Second Edition. Taylor and Francis, New York, NY.

7. Moore and McCabe 1989, Introduction to the Practice of Statistics. W.H. Freeman & Company, New York, NY.

8. Nocker, A., Sossa, K.E. and Camper, A.K. (2007) Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *Journal of Microbiological Methods* 70, 252-260.

9. Sambrook, J., and D. W. Russell. 2001. Molecular Cloning: A Laboratory Manual, 4th ed, vol. 1-3. Cold Spring Harbor Laboratory Press, New York.

10. Siefring, S., M. Varma, E. Atikovic, L. Wymer and R.A. Haugland R.A. 2008. Improved real-time PCR assays for the detection of fecal indicator bacteria in surface waters with different instrument and reagent systems. J. Water and Health. 6, 225-237.

11. SWGDAM. 2004. Revised Validation Guidelines: Scientific Working Group on DNA Analysis Methods. Forensic Science Communications 6.

12. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 63:3741-3751.

13. Wittwer, C.T., and N. Kusukawa. 2004. Real-time PCR, p. 71-84. In D.L. Persing, F.C. Tenover, J. Versalovic, Y-W. Tang, E.R. Unger, D.A. Relman, and T.J. White (ed.), Molecular Microbiology, Diagnostic Principles and Practice. ASM Press, Washington D.C.

Appendix D – Guidelines for the Validation of Efficacy Test Methods

Introduction

EPA regulates antimicrobial pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Antimicrobial pesticides are substances used to kill or suppress the growth of harmful microorganisms on inanimate objects and surfaces. They are divided into two categories: Non-public health and public health products.

The Agency has waived requirements to submit efficacy data for registration of non-public health antimicrobial products. However, each applicant and registrant must ensure through laboratory testing that products are efficacious, and maintain the data on file if the Agency requests its submission. Registration of public health antimicrobial products, on the other hand, requires submission of efficacy data to support each label claim and use pattern.

1.0 Selection of Test Method to Generate Product Efficacy Data

Efficacy requirements of antimicrobials are set forth in the 40 Code of Federal Regulations (CFR) Part 158. Guidance documents specific for efficacy testing of antimicrobials are found in Registration Policy Documents and Disinfectant Technical Science Section (DIS/TSS) and are posted on the Agency's Web site (www.epa.gov/oppad001). The guidance documents recommend specific test methods and performance standards (e.g., pass/fail criteria) for each efficacy claim (e.g., disinfectant, sanitizer, sterilant, etc.). Use of standardized, validated test methods are preferred by the Agency, but not required. Validated methods are preferred because the method's performance was evaluated and deemed suitable for its use (e.g., suitable for efficacy evaluation of liquid products on hard surfaces). Most of the efficacy test methods are archived and managed by AOAC International, a standard-setting organization. In most cases, an AOAC method is validated according to strict guidance provided by AOAC, and official changes or modifications to a method can only be approved under AOAC purview. AOAC methods have been in place for several decades and are primarily qualitative in nature (i.e., presence or absence of viable test organism following exposure to test chemical). For example, data generated using the AOAC Use Dilution Methods, the AOAC Germicidal Spray Products Method, and the AOAC Sporicidal Activity of Disinfectants Method support certain claims for public health pesticides and are considered critical to the Agency's decision making process in registering antimicrobial pesticides. Other currently-recommended methods are those that the Agency believes have historically proven to be well-developed and suitable for their intended use (e.g.,

ASTM standards) but not necessarily subjected to multi-laboratory validation studies. Currently, the Agency is promoting the development of quantitative (i.e., kill measured as a log reduction) rather than qualitative efficacy tests and is spearheading improvements to existing qualitative and quantitative methods to further enhance method performance. In addition, the Agency is currently working to harmonize antimicrobial efficacy test methods internationally. For efficacy claims for which no recommended/standard test method exists (e.g., biofilm disinfection) or for instances in which a registrant believes a new protocol may better demonstrate efficacy of a product than the standard method, registrants may submit protocols to the Agency for review prior to data collection. The review process includes an extensive in-house and optional expert panel protocol review, requirements for independent verification of the protocol in three separate laboratories and demonstration of statistical validity (www.epa.gov/oppad001/efficacyproto.htm). For all new methodology, the registrant, through EPA's protocol review process, will have to provide historical evidence (i.e., data) that the method is reproducible and relevant for its intended use.

2.0 Validation of Efficacy Test Methods and Study Plan Preparation

Although EPA does not currently require validated methodology, the user community (i.e., registrant, government agencies) may determine that there is a need for determining method performance across laboratories and may seek validation. EPA is interested in rigorous method validation to ensure that the Agency and its stakeholders have the means to generate data that are accurate and reliable. The conventional approach of validating a method through collaborative study under the auspices of a third party, standard setting organization such as AOAC International is highly desirable, but is not required.

Historically, EPA recognizes and recommends the use of many AOAC International methods because of the standard setting organization's validation process. AOAC International has a well-structured validation program (Official Methods Program) that evaluates methods through interlaboratory collaborative studies (minimum of 8-10 labs), and provides a benchmark for the method validation process. Multi-lab collaborative studies are used to determine key performance indicators of the method, including between and within-lab variability and ruggedness. In advance of submitting a protocol for validation, it is advisable to generate in-house validation data or arrange for an independent lab to validate the method. For more

information on AOAC International's method validation program, consult their Web site at www.aoac.org.

Method validation may also be achieved by individuals who wish to conduct a collaborative study and publish the results in a peer-reviewed scientific journal, without participation in a formal third party validation program such as the AOAC Official Methods Program. For scientists interested in pursuing method validation independently, the AOAC Web site is an excellent source of information on collaborative study design and data analysis (see the Official Methods Program Manual at www.aoac.org/vmeth/omamanual/omamanual.htm).

Once a method is validated and published, it is important that it be updated on a periodic basis. Methods are not static. Over time, vendors and technologies change, and new formulations or surfaces are introduced, necessitating revisions of standard methods.

3.0 Pre-Validation Considerations: Factors Affecting Efficacy of Antimicrobial Products

Springthorpe and Sattar (2005) provide an excellent overview of factors affecting the antimicrobial activity of products. An understanding of these factors is crucial to the development of an efficacy test method that is to be subjected to validation. Below is a brief summary of the factors described by Springthorpe and Sattar (2005):

- X Formulation – minimum concentration of active ingredients required for efficacy; different inert ingredients have different affects on efficacy of the product.

- X Target organism/organic soil load – Microorganisms vary in their ability to survive exposure to antimicrobials. In a healthcare environment, microorganisms may be found in blood and other body fluids (organic soil) present on surfaces, making them more difficult to kill.

- X Temperature – Product label instructions regarding temperature must be provided, as efficacy generally increases with increase in temperature.

- X Product diluent – Hard water can decrease efficacy of a diluted product. For products without hard water claims, labels rarely specify the type of water to use for dilution. Distilled water was typically the diluent used in efficacy testing performed to support product registration.

- X However, product users, without specific label guidance, are more likely to use tap water than distilled water to dilute the product. Tap water varies in hardness and the user may unintentionally reduce the efficacy of the product by using tap water.

- X Contact time – Treated surfaces must remain wet for a minimum of the label-specified contact time for the product to be effective.

- X Carrier surface – Microorganisms must be eliminated from a variety of surfaces (e.g., steel, glass, wood) in the environment. Carriers used in efficacy tests must effectively simulate the surfaces to be treated by an end user.

- X Precleaning agents – Many antimicrobial product labels specify pre-cleaning of the surface to be treated. Some cleaning agents may inhibit the efficacy if not rinsed effectively from the surface prior to application of the antimicrobial.

- X Method of application – Different methods of application (e.g., mop, cloth, sprayers) result in different amounts of product applied to the surface to be treated. Amount applied per surface area may affect efficacy.

- X Storage and shelf life – Conditions and length of storage of a product may adversely affect efficacy. Once diluted, a product's potency may decrease more rapidly than with the concentrated product.

- X pH/humidity – Antimicrobial products work best at specified pH and humidity levels. Product labels should provide guidance as to optimal pH and humidity levels for product use.

In addition to the factors discussed by Springthorpe and Sattar (2005), there are the following factors that influence the outcome of an efficacy evaluation of an antimicrobial product:

- X Inoculum titer of microorganisms present in the test system – Many current EPA-recommended efficacy test methods are qualitative (presence/absence of viable microbes after treatment) rather than quantitative. Consequently, tests may vary in the number of organisms present on a carrier or in the test system. Depending upon the sensitivity of a method, the outcome of efficacy evaluation may be affected by a variable population of microorganisms in the test system.

- X Quality of microorganisms present in the test system – Currently, there is no established standard (i.e., chemicals/disinfectants) for use in efficacy test methods. Consequently, for decades, scientists have relied upon AOAC efficacy test standards such as phenol resistance testing or HCl testing to estimate the intrinsic resistance of test microbes to disinfectants (indicator of suitable organism population). In 2001 (PR

Notice 2001-04; http://www.epa.gov/opppmsd1/PR_Notices), EPA determined that phenol resistance testing was an unsatisfactory standard for determining organism hardiness and recommended a minimum inoculum level of 10^4 organisms/carrier for AOAC carrier based efficacy tests. HCl testing still remains a required component of the AOAC Sporicidal Activity Test as an indicator of spore hardiness. The quality of the microorganisms used in an efficacy evaluation affects the outcome of testing—use of less resistant organisms may make the product appear more efficacious than it actually is.

X Technique-sensitive procedures – Many of the recommended efficacy methods may contain technique-sensitive steps. It is critical that scientists performing the tests be trained in the conduct of the method. Following the method exactly is crucial for proficiency and validity of test results.

4.0 Desirable Attributes of a Validated Test Method

In addition to the criteria for method selection described Section 2.2 of the main document, the following are desirable components of and recommendations for an efficacy test method that is to be subjected to validation:

X Suitable for use with the test microorganisms;

X Protocols for culturing/enumerating test microorganism;

X Acceptable statistical profile addressing within and between lab variability (Tilt and Hamilton 1999);

X Suitable for multiple active ingredients (i.e., different formulations);

X Contains a percent recovery/minimum detection level;

X For carrier-based tests, includes inoculation procedure, method of determining populations of microorganisms on a carrier, and a target range for the microorganism population;
- S Includes a neutralization confirmation procedure;
- S Addresses means for managing contact time and temperatures (i.e., includes references to calibrated timers and thermometers); and
- S Prior to pursuing method validation, knowledge of the target performance standard is essential. For example, if a 6-$\log_{10}$ kill of the target organism on a porcelain carrier is required for sporicidal decontamination products, then the efficacy method must be able to accommodate a minimum of a 7-$\log_{10}$ challenge per carrier.

5.0 Method Performance Characteristics

The method performance characteristics for efficacy testing remain the same as described in Section 2.4 of the main document. Accuracy defined as measure of the overall agreement (e.g., pass or fail efficacy) to a known value, does not apply to efficacy testing since there are currently no established standards (i.e., chemicals/disinfectants) available for use in efficacy tests. Accuracy is measured from results of repeating the test several times and by comparison to an existing method.

6.0 References

1. Springthorpe, V.S. and Sattar, S.A. (2005) Carrier Tests to Assess Microbiological Activities of Chemical Disinfectants for Use on Medical Devices and Environmental Surfaces. J. AOAC Int. 88, 182-201.

2. Tilt, N. and Hamilton, M.A. 1999. Repeatability and Reproducibility of Germicide Tests. J. AOAC Int. 82, 384-389.

Appendix E – The Method Approval Process

Once validated, a method can receive Agency approval and be designated as an EPA method. There are several ways that methods can be approved; these approaches are outlined below.

Method Approval through the Rulemaking Process

The rulemaking process begins with EPA publication of a proposed rule in which the agency cites a method or methods that it plans to use to implement the rule. EPA then solicits and receives comments during a public comment period. Once these comments are addressed and the Agency promulgates the final rule, the method identified in the rule is considered approved and ready for use. The method in the rule is then considered to be a reference method.

Until recently, new or modified methods under the Alternate Test Procedures (ATP) program also followed the same approval and rule making process described above (see Section 1.1 of this appendix). Thus, any modified or new method may take several years before it becomes a final rule.

1.0 Alternate Method Approval Processes

After a reference method has been established, alternate methods that are easier, less expensive, or more accurate often become available. The possible approval processes for these alternate methods are described below.

1.1 Alternate Test Procedure (ATP) Program

The ATP program is another avenue to allow emerging technologies that reduce cost and enhance data quality. Under this program, all modifications that are not explicitly allowed by a method cited in a rule require prior EPA approval through the ATP program. An ATP can be a modified method or a new method. The applicant(s) of a particular ATP can only submit an application to the ATP program after the method has undergone performance characterization.

2.0 Expedited Methods Approval

Due to the lengthy time required for new or modified method approval, EPA has recently developed the expedited method approval notice. To use this process, EPA must have already promulgated at least one analytical testing method for the analyte or microorganism through the

rulemaking process. Section 1401 of the SDWA allows EPA to approve additional testing methods through this expedited approval that simply involves publishing the alternative method in the Federal Register (Charlton *et al.* 2000). Therefore, the new or modified methods submitted through the ATP program can now be approved by this expedited approval process. However, the performance equivalence of the new testing method to the reference method (2008, FR 73: 31616) must be demonstrated before being considered for approval by the expedited process. A process flow diagram illustrating the approval steps for undertaking an ATP and its relationship to the reference method is provided in Figure E-1.

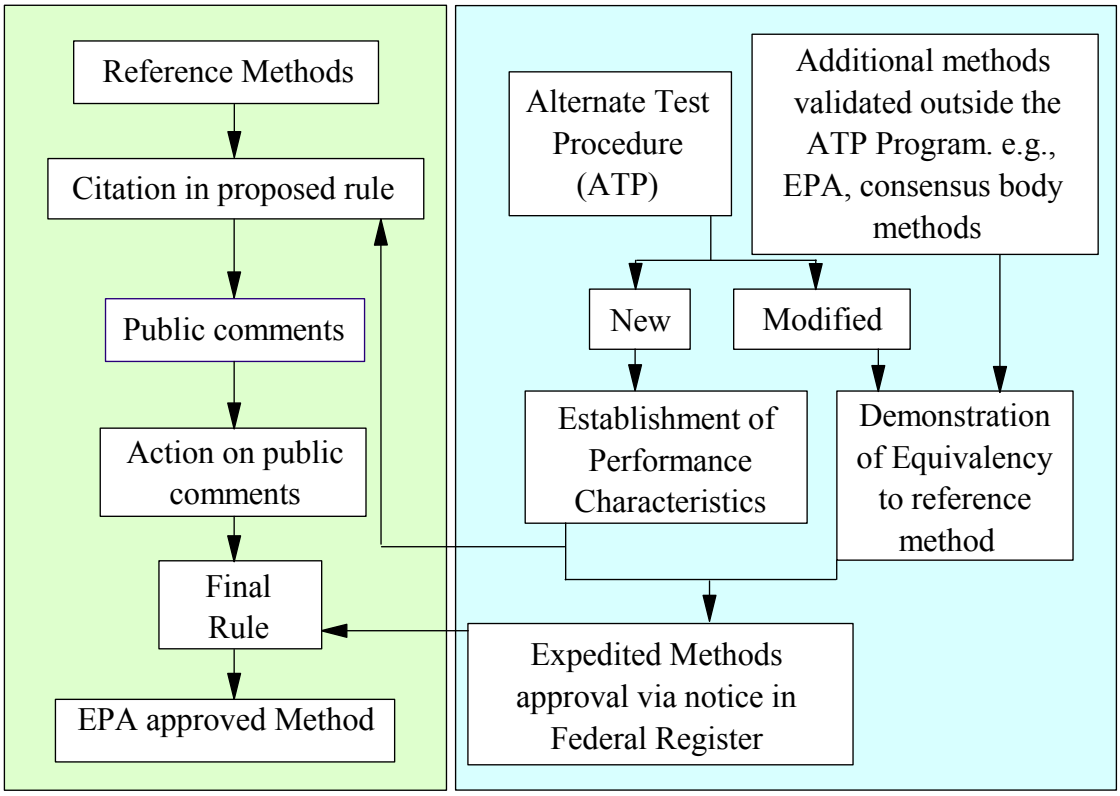


Figure E-1. Methods Approval Schematic Representing Reference Methods and Alternate Test Procedures

3.0 Reference Methods and Other Approved Methods

EPA designates an approved method as the "reference method" for each combination of analyte and technique. Any newly developed method that contains a unique combination of analyte and technique is considered a new method and, when approved, can be designated as the

reference method for that unique combination of analyte and technique. Any approved method not designated as a reference method has been designated as an "other approved method." All methods must contain standardized quality control (QC) tests.

The person or organization that develops a reference method for a particular combination of analyte and technique is responsible for validating the method and for developing the QC acceptance criteria. QC acceptance criteria are based on data generated during the method validation study.

4.0 References

1. Charlton, S., R. Giroux, D. Hondred, C. Lipton, and K. Worden. 2000. PCR Validation and Performance Characteristics - AEIC Biotech Consensus Paper. Analytical Environmental Immunochemical Consortium.