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Standard Guide for Assessment of Antimicrobial Activity Using a Time-Kill Procedure[1]

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1. Scope

1.1 This guide covers an example of a method that measures the changes in a population of aerobic microorganisms within a specified sampling time when antimicrobial test materials are present. ~~Several options for organism selection and growth, inoculum preparation, sampling times and temperatures are provided. When the technique is performed as a specific test method, it is critical that the above mentioned variables have been standardized. Antimicrobial activity of specific materials, as measured by this technique, may vary significantly depending on variables selected. It is important to understand the limitations of in vitro tests, especially comparisons of results from tests performed with different parameters. As an example, test results of microorganisms requiring growth supplements or special incubation conditions may not be directly comparable to organisms evaluated without those stated conditions.~~

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1.1.3 Antimicrobial activity of specific materials, as measured by this technique, can vary significantly depending on variables selected.

1.1.4 Test Method E2783 may be referenced as an example of using fixed conditions and set variables to evaluate antimicrobial efficacy of water-miscible compounds.

1.1.5 This guide serves as a general teaching document for evaluating the antimicrobial activity using a variety of conditions to offer the flexibility needed in test conditions to cover a broad range of microorganisms and test substances.

1.1.6 It is important to understand the limitations of *in vitro* tests, especially comparisons of results from tests performed with different parameters. As an example, test results of microorganisms requiring growth supplements or special incubation conditions may not be directly comparable to organisms evaluated without those stated conditions.

1.2 Knowledge of microbiological techniques is required for this procedure.

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

[1] This guide is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

E1054 Practices for Evaluation of Inactivators of Antimicrobial Agents

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

E2783 Test Method for Assessment of Antimicrobial Activity for Water Miscible Compounds Using a Time-Kill Procedure

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of standard terms relating to antimicrobial agents used in this guide, refer to Terminology E2756.

3.2 ~~*Definitions:*~~*Definitions of Terms Specific to This Standard:*

3.2.1 *inoculum suspension, n*—the initial suspension of test organism used to inoculate the test material. This may also be known as the organism inoculum (see 8.3).

3.2.2 *microbial population, n*—the microbial count (cfu/mL) in the final volume of test material (see 9.4). This may also be known as the "numbers control." The measurement may be taken at time zero which may be termed "Initial Population." Alternatively, the measurement may be taken at each exposure time or the longest exposure time used during testing to simulate the test procedure which may be termed "Final Population."

3.2.3 *neutralization, n*—the process for inactivating or quenching the activity of a test material. This may be achieved through physical means (for example, filtration, dilution) and/or the addition of chemical agents, called neutralizers.

3.2.4 *neutralizer, n*—a chemical agent used to inactivate, neutralize, or quench the microbicidal properties of an antimicrobial agent.

3.2.5 *total test volume, n*—the volume of test material plus the volume of inoculum suspension.

4. Summary of a Basic Test Method

4.1 The test material or a dilution of the test material is brought into contact with a known population of microorganisms for a specified period of time at a specified temperature. An appropriate and specified neutralization technique is applied to quench the antimicrobial activity of the test material at specified sampling intervals (for example, 30 s, 60 s, or any range covering several minutes or hours), and the surviving microorganisms are enumerated. The percent and/or $\log_{10}$ reduction is calculated by comparison with the microbial population.

5. Significance and Use

5.1 This procedure may be used to assess the *in vitro* reduction of a microbial population of test organisms after exposure to a test material.

6. Apparatus

6.1 *Sterile Vials or Test Tubes,* or equivalent.

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

6.2 *Timer (Stop-clock)* that displays minutes and seconds.

6.3 *Water Bath, Controlled Temperature Chamber,* or equivalent capable of maintaining test system at the specified exposure temperature ~~±2°C.~~±2 °C.

6.4 *Colony Counter,* any of several manual or automated types may be used.

6.5 *Incubator,* any capable of maintaining a specified temperature ~~±2°C~~±2 °C may be used.

6.6 *Sterilizer,* any steam sterilizer capable of producing the conditions of sterilization.

6.7 *Vortex Mixer, Magnetic Stirrer,* or equivalent.

6.8 *Spiral Plating System,* (optional).

6.9 *Sterile Bacteriological Pipettes,* for viscous test materials, positive displacement pipettes or syringes may be necessary.

6.10 *Water Dilution Bottles,* any sterilizable container having appropriate capacity and tight closures may be used.

6.11 *Sterile Cotton Applicator Swabs*.

7. Reagents and Materials

7.1 *Dilution Fluid or Diluent*—sterile water, 0.9 % (w/v) saline, sterile Butterfield's buffered phosphate diluent,[3] or equivalent.

7.2 *Broth Growth Medium*—soybean-casein digest broth or equivalent liquid media appropriate to supporting growth of the test organism(s), with appropriate neutralizers, if required (see ~~3.1~~3.2).

7.3 *Solid Growth and Plating Medium*—soybean-casein digest agar[4] or equivalent solid media appropriate to support growth of the test organism(s), with appropriate neutralizers, if required (see ~~3.1.3~~3.2.3 and ~~3.1.4~~3.2.4).

7.4 *Sterile Deionized Water,* or equivalent (Specification D1193, Type III).

8. Test Organism Preparation

8.1 The test organism selected may be representative of the microbial flora encountered under the conditions of use of a test material or may be standardized strains.

8.2 *Organism Preparation*—Transfer culture(s) from stock twice (once every 18 to 24 h or as appropriate for the test organism) into appropriate growth medium to maintain the organism in growth phase. The second transfer may be made into a volume of growth medium that will provide a microbial suspension sufficient to conduct testing and controls. Consider that additional volume may be needed to permit testing of multiple samples or time points.

8.2.1 Alternatively, the transfers may be made onto agar plates or slants, and the inoculum suspension prepared by washing the organism from the slant with an appropriate broth or diluent.

NOTE 1—Reports in the published literature have noted differences in microbial kill or susceptibility as a result of different propagation methods. It is recommended that tests be conducted using a consistent procedure for organism propagation.

[3] Horowitz, W., Ed., *Official Methods of Analysis of the AOAC, 18th Ed.*, Association of Official Analytical Chemists, Washington, DC, 2000; Journal of the Association of Official Analytical Chemists. Vol 22, No. 635, 1939.

[4] *U.S. Pharmacopeia, 38–NF33*, The United States Pharmacopeial Convention, Inc. Rockville, MD, 2000.

8.3 *Inoculum Suspension Preparation and Determination of the Microbial Population:*[5]

8.3.1 To prepare inoculum suspension directly from broth growth medium, a dilution in sterile broth (diluent is same as that used for broth growth medium) may be performed to achieve the desired concentration.

8.3.2 To prepare inoculum suspension in dilute broth, an up to 1:10 dilution of the suspension into Butterfield's buffered phosphate diluent or equivalent may be performed to reduce the concentration of the growth medium.

8.3.3 Inoculum suspensions in broth may be diluted or concentrated to achieve the desired concentration or they may be centrifuged and reconstituted in Butterfield's buffered phosphate diluent, broth, saline, or equivalent, to achieve the desired concentration.

8.3.4 To prepare the inoculum suspension from an agar plate or slant, wash microbial growth or transfer the growth aseptically using a sterile swab from the agar surface with Butterfield's buffered phosphate diluent, saline, or equivalent.

NOTE 2—Because certain antimicrobials (for example, alcohol and iodine) are sensitive to organic material and may have activity reduced by even the slightest organic load, washed inoculum suspensions, whether established initially in broth or from solid media, may be used.

8.3.5 The inoculum suspension should be prepared to achieve a minimum population concentration of 10^6 cfu/mL (see 9.4). The final inoculum suspension should be well-mixed prior to transfer to test material (see 9.5).

8.3.6 The inoculum suspension should be plated in duplicate by standard microbiological procedures at the initiation and completion of testing. Appropriate dilutions should be prepared and enumerated by standard microbiological procedures (spread- or pour-plating, microbial filtration, or spiral-plating). The initial and final titer of the inoculum should be within ± 0.5 $\log_{10}$ for a valid test. This step may be omitted where Microbial Population enumeration is conducted.

8.4 To perform the Microbial Population (~~3.1.2~~3.2.2) quantitation, a volume of inoculum suspension equivalent to that inoculated into the test material is added to a dilution blank containing the same volume as used for the test material. To simulate the test recovery fluid, the inoculated dilution blank is serially diluted and neutralized in the same manner as the test material. The Initial Population and Final Population counts must be within ± 0.5 $\log_{10}$ for a valid test.

NOTE 3—Depending on the microorganism being tested and the duration of the experiment, verifying the Final Population may not be necessary. It is recommended to confirm that there is not a loss of viability over the amount of time required for a given experiment (for example, 1 to 2 h) for a particular organism, but not necessary to perform each time for repeat experiment using the same conditions.

8.4.1 Incubate plates at the specified temperature ~~±2°C~~±2 °C for 24 to 48 h or as appropriate for a test organism.

8.4.2 Count colonies and record raw data as cfu/plate to determine the number of surviving organisms. Average duplicate plate counts (2 plates from each dilution) and multiply by the dilution factor to calculate cfu/mL of inoculum.

9. Basic Procedure

9.1 Select the concentrations of the test material to be tested. Each concentration is tested in duplicate. Each recovery sample is plated in duplicate. See Fig. 1.

9.2 Prepare each test concentration in duplicate. Dilutions should be prepared using sterile distilled water. Other diluents, such as saline or a buffer, may be used for informational purposes or if test material is typically diluted that way under conditions of use. Ensure that the test material is completely dispersed. Some test materials may require gentle heating before they become completely dispersed. Allow the solutions to equilibrate to 25 ± ~~2°C.~~2 °C.

NOTE 4—Additional test temperatures may be considered based on the intended use of the test material (for example, 22 ± ~~2°C~~2 °C for room temperature; 30 ± ~~2°C~~2 °C for temperature of human skin; and 38 ± ~~2°C~~2 °C for temperature of "warm" water). A solid test material may require warming to and holding at 45 ± ~~2°C~~2 °C to disperse the test material and maintain uniformity during testing. Under no circumstances should a test temperature be chosen when the temperature effects, alone, cause microorganism death.

[5] Brown, M. R. W., Gilbert P., *Microbiological Quality Assurance: A Guide Towards Relevance and Reproducibility of Inocula*, CRC Press, New York, NY, 1995.

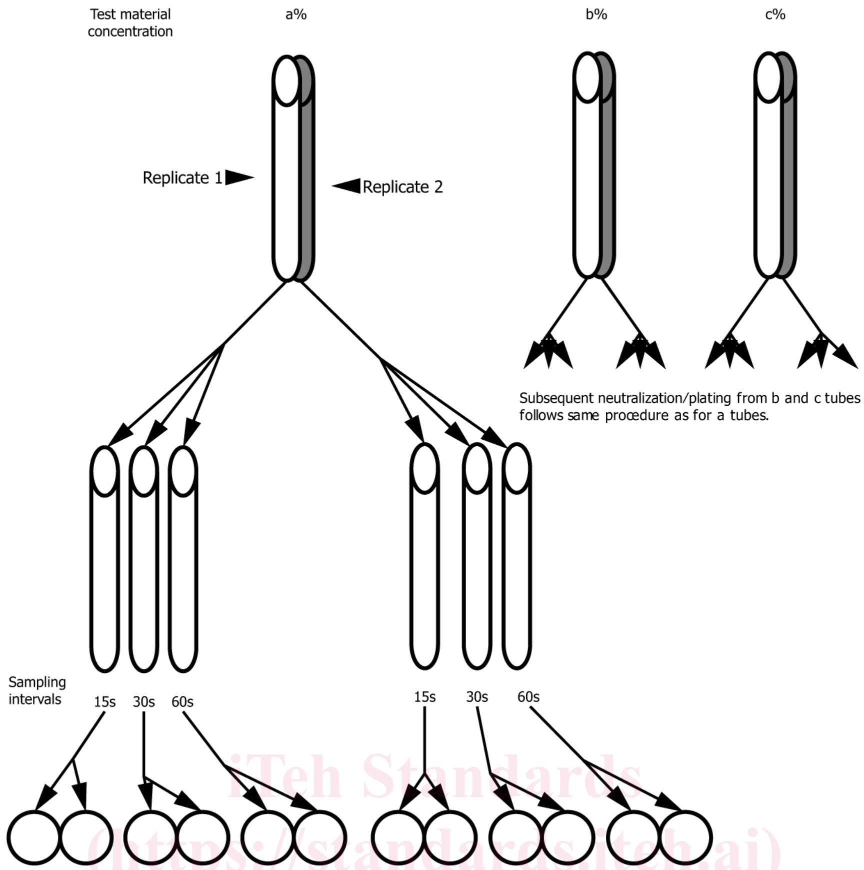

FIG. 1 Time-Kill Dilution/Plating Scheme

9.3 For selection of contact times, a minimum time period should be selected based on the ability to perform test sampling reproducibly in this time frame (for example, 15, 30, or 60 s). Other time points may be selected based on the intended use of the test material, or several over a period of time in order to construct a curve.

9.4 To minimize buffer interference and reduction of antimicrobial activity, the volume of inoculum suspension should be less than or equal to 5 % of the total test volume. The microbial population or numbers control should achieve a minimum of 10^6 cfu/mL of the test organism.

NOTE 5—The formulation may be prepared at a higher concentration to account for the dilution from the inoculum suspension to achieve the proper final concentration. For example, if the 4 mL of test sample will be combined with 1 mL of the inoculum suspension in buffer, the test sample should be prepared at 1.25X concentration.

9.5 The inoculum suspension should be uniformly mixed. Begin mixing the test sample, transfer inoculum suspension to the sample and the control blank, and maintain mixing to disperse the inoculum suspension. Uniform mixing throughout the test is crucial for test repeatability. As applicable, mix the test mixture with care to minimize foam formation. The formation of foam may cause anomalous results.