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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.

1.1.1 Several options for organism selection and growth, inoculum preparation, sampling times and temperatures are provided.

1.1.2 When the technique is performed as a specific test method, it is critical that the above mentioned variables have been standardized.

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.

1.4 *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, health, and environmental practices and determine the applicability of regulatory 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 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

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[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.

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.

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.

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 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 3.2).

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 3.2.3 and 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.

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

[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.

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