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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 examples of a basic method to measure the changes of a population of aerobic microorganisms within a specified sampling time when tested against antimicrobial test materials *in vitro*. Several options for organism selection and growth, inoculum preparation, sampling times and temperatures are provided. When the basic technique is performed as a specific test method it is critical when evaluating the results to ensure that such 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 under different circumstances. As an example, test results of microorganisms requiring growth supplements, or special incubation conditions, may not be directly comparable to more robust organisms under the conditions of a single procedure.

1.2 Knowledge of microbiological techniques is required for this test.

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 may involve hazardous materials, operations and equipment. 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 practices and determine the applicability of regulatory requirements prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

3. Terminology

3.1 *Definitions:*

3.1.1 *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 8.2).

3.1.2 *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 "initial population" or "numbers control."

3.1.3 *neutralization, n*—a process which results in the inactivation or quenching of the antimicrobial activity of a test material. This may be achieved through dilution of the test material(s) or with the use of chemical agents, called neutralizers, to reduce or quench the antimicrobial activity.

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

3.1.5 *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. The activity of the test material is quenched at specified sampling intervals (for example, 30 s, 60 s, or any range covering several minutes or hours) with an appropriate neutralization technique. The test material is neutralized at the sampling time and the surviving microorganisms enumerated. The percent or $\log_{10}$ reduction, or both, from either an initial microbial population, or test blank is calculated.

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.

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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.

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

6.3 *Shaking Water Bath or 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 incubator capable of maintaining a specified temperature ±2°C may be used.

6.6 *Sterilizer,* any suitable 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.

7. Reagents and Materials

7.1 *Dilution Fluid or Diluent,* sterile water, 0.65 % saline, sterile Butterfield's buffered phosphate diluent[3] or equivalent.

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

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

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

8. Test Methods

8.1 *Test Organisms:*

8.1.1 The test organisms selected may be representative of the microbial flora encountered under the conditions of use, or may represent standardized strains. The organism should be capable of providing reproducible results under specific test conditions.

8.1.2 *Organism Preparation*—Transfer culture(s) from stock twice (once every 18 to 24 h or as appropriate for the test organism) into appropriate growth media. The second transfer may be made into a volume of growth medium to produce sufficient microbial suspension to inoculate. Volumes used should permit testing of multiple samples or time points.

8.1.2.1 Alternatively, the transfers may be made onto agar plates or slants and the inoculum suspension may be 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 antimicrobial resistance as a result of cell protection in broth or as a result of washing cells. It is recommended that tests be conducted with either all cells prepared in broth dilutions or with all cells prepared by washing.

8.2 *Inoculum Suspension Preparation and Determination of the Microbial Population or Numbers Control:*[5]

8.2.1 To prepare inoculum suspension directly from broth, a dilution in sterile broth (the same as that used for growth medium) may be performed to reduce the concentration of the microorganisms to the appropriate level.

8.2.1.1 To prepare inoculum suspension in dilute broth, a 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.

8.2.1.2 Inoculum suspensions grown from broth may be diluted to appropriate concentration or they may be centrifuged and reconstituted in Butterfield's buffered phosphate diluent, broth, saline, or equivalent, to the appropriate concentration.

8.2.2 To prepare the inoculum suspension from an agar plate or slant, wash microbial growth from the agar surface with Butterfield's buffered phosphate diluent, saline, or equivalent.

NOTE 2—Antimicrobials sensitive to organic material (for example, alcohol and iodine) may have reduced activity by even the slightest organic load and therefore thoroughly washed inoculum suspensions only, whether grown initially in broth or from solid media, should be used.

8.2.3 The inoculum suspension should be prepared to achieve a minimum of 10^6 cfu/mL microbial population (see 9.4). Results of tests where the initial microbial populations differ from the test population by greater than $2\log_{10}$ should be interpreted with care because of the exponential nature of the populations. The final inoculum suspension should be well mixed prior to addition to test materials (see 9.5).

8.2.4 The inoculum suspension should be enumerated in duplicate by standard microbiological procedures at the initiation and completion of testing. Appropriate dilutions are prepared and enumerated by standard microbiological procedures (Spread or pour plating, microbial filtration, or spiral plating). The initial and final count of the inoculum should be within ± 0.5 $\log_{10}$ for a valid test.

8.2.4.1 To perform the population quantitation of the control blank, 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. The initial and final count of the population in the blank must be within ± 0.5 $\log_{10}$ for a valid test.

8.2.5 Incubate plates at the specified temperature ±2°C for 24 to 48 h or as appropriate for the test organism(s).

8.2.6 Count colonies and record raw data as cfu/plate to determine surviving organisms. Average duplicate plates (2

[3] Horowitz, W., Ed., *Official Methods of Analysis of the AOAC, 17th Ed.*, ch. 17, p. 4, sec. 17.2.01, A(m), Association of Official Analytical Chemists, Washington, DC, 2000; (As cited in, Butterfield's Phosphate Buffer, Journal of the Association of Official Analytical Chemists. Vol 22, No. 635, 1939.)

[4] *U.S. Pharmacopoeia, 24th Revision*, The United States Pharmacopoeia 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.