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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.

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 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

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 *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).

3.1.2 *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 "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."

3.1.3 *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.

3.1.4 *neutralizer, n*—a chemical agent used to inactivate, neutralize, or quench the microbicidal 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. 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.

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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.

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 3.1).

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 and 3.1.4).

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 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) 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. The Initial Population and Final Population counts must be within ±0.5 $\log_{10}$ for a valid test.

[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.

8.4.1 Incubate plates at the specified temperature ±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.

NOTE 3—Additional test temperatures may be considered based on the intended use of the test material (for example, 22 ± 2°C for room temperature; 30 ± 2°C for temperature of human skin; and 38 ± 2°C for temperature of "warm" water). A solid test material may require warming to and holding at 45 ± 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.

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.

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.

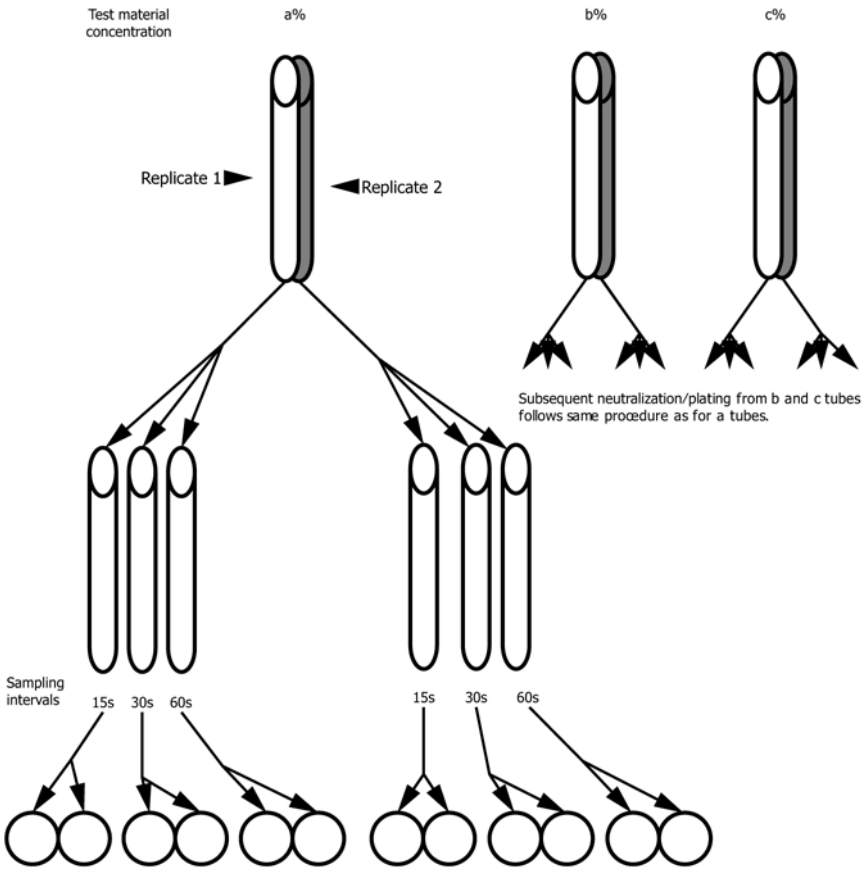

FIG. 1 Time-Kill Dilution/Plating Scheme

NOTE 4—Methods of mixing include use of a vortex mixer, stomacher, syringe, positive displacement pipette, constant velocity mix plate with stir bar or other methods that will rapidly disperse inoculum into the test material, while maintaining uniform mixing of the test mixture. Viscous materials may present difficulty in mixing.

9.6 At predetermined time intervals, remove an aliquot, typically 1 mL, and make appropriate dilutions in sterile Butterfield's buffered phosphate diluent or equivalent containing appropriate neutralizers, if needed (see 3.1.3 and 3.1.4). See Test Methods E1054 to confirm the neutralization technique. See Fig. 1.

9.7 Recover viable organisms from appropriate dilutions by culturing in duplicate (see 7.3) using spread- or pour-plating, microbial filtration, spiral-plating, or other valid microbial recovery methods. See Fig. 1.

9.8 Incubate plates at the specified temperature ±2°C for 24 to 48 h or as appropriate for each organism selected. Incubation time should allow growth of surviving organisms, without overgrowth of colonies, which makes enumeration difficult.

9.9 To determine surviving organisms, count colonies and record raw data as cfu/plate. Average duplicate plate counts (2 plates from each replicate dilution) and multiply by the dilution factor to arrive at cfu/mL of test suspension. This averaged count should then be converted to $\log_{10}$ scale.

9.10 *Formula for Calculating Microbial* $\log_{10}$ *Reduction:*

Step 1: Transform the measured Initial and Final Populations, Inoculum Suspension, and Test recoveries (see 8.4.2) to $\log_{10}$ scale.

Step 2: If more than one tube was used for determining the population, transform each measured value to $\log_{10}$ scale, then calculate the geometric mean and variance associated with the use of multiple tubes.

Step 3: Calculate the $\log_{10}$ reduction for each test substance concentration at each exposure:

$$\text{Log}_{10}\text{ reduction (LR)} = \text{mean log}_{10}\text{ (surviving microbial population)}$$

$$\text{Log}_{10}\text{ Reduction (LR)} = \text{mean log}_{10}\text{ (Microbrial Population)} - \text{mean log}_{10}\text{ (surviving test population)}$$

Step 4: Calculate the standard error of the mean:

$$\text{Standard Error} = (\text{variance of the log}_{10}\text{ surviving microbial population/number of replicates})$$

$$\text{Standard Error} = \sqrt{[(\text{Variance of log}_{10}\text{ Microbial Population/No. of Replicates}) + (\text{Variance of log}_{10}\text{ surviving test population/No. of Replicates})]}$$

NOTE 5—The above calculations for standard error assess only the with-in experiment variability.

9.11 *Calculate the Percent Reduction:*

$$\text{Percent Reduction (\%)} = 100 \times (1 - 10^{-LR})$$

10. Procedure Controls

10.1 *Purity Control*—Conduct an isolation streak of the test strain to verify culture purity. Incubate alongside the test.

10.2 *Sterility Control:*

10.2.1 Conduct sterility controls for all reagents including diluents, growth media, and neutralizers. Incubate alongside the test.

10.2.2 For agar or broth media, incubate a plate or tube of media. For diluents or neutralizer solutions, add at least 0.1 mL to an appropriate agar plate.

10.3 *Neutralization Control*—Conduct a neutralization control alongside or prior to testing in accordance with Test Methods E1054 to assure the recovery technique and neutralization/growth media used effectively and immediately neutralize any residual test substance in the recovered population.

11. Report

11.1 The report should contain at a minimum, the following information:

11.1.1 *Test Organism*—Specify the genus, species, origin, growth temperature, transfer frequency and incubation period. Describe the rationale for selection of the test organism(s).

11.1.2 *Media*—Specify the growth and plating media (broth/agar).

11.1.3 *Preparation of the Test Organism*—Specify the organism recovery method, including dilution and centrifugation conditions if applicable.

11.1.4 *Initial Microbial Population*—State the microbial population at the initiation and completion of testing.

11.1.5 *Control Blank*—Specify the make-up of the control blank (for example, water, saline).

11.1.6 *Enumeration*—Specify recovery method, including technique, media, incubation conditions, and special circumstances (for example, anaerobic, and/or CO_2 incubation).

11.1.7 *Neutralization*—Specify neutralization technique employed and provide data demonstrating successful neutralization (see 3.1).

11.1.8 *Data*—Show all raw data and calculations.

12. Precision and Bias

12.1 *Precision*—The precision of the practice will depend upon the selection of test organisms, growth media, and test materials evaluated. These sources of variation should be considered when testing antimicrobial performance.

12.2 *Bias*—The bias of this practice will be affected by the same variables listed in 12.1. Two additional variables depend on the specific procedures used to evaluate a test material.

12.2.1 Each CFU is assumed to originate from a single cell of a microbe. In reality, microbes often form aggregates, which can result in a single colony. Consequently, viable count data are likely to underestimate the total number of viable organisms in a sample.

12.2.2 The metabolic state of individual microbes may be affected by numerous physical-chemical variables in the growth media. Injured cells or cells that have relatively long generation times may not form colonies within the time allotted for test observations. This will result in an underestimation of the numbers of viable microbes in a sample.

13. Keywords

13.1 antimicrobial; *in vitro*; $\log_{10}$ reduction; percent reduction; time-kill

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