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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 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.

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:*

D 1193 Specification for Reagent Water

E 1054 Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfectant, Sanitizer, Antiseptic or Preserved Products

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.

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

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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[2] 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,[3] 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 D 1193, 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:*[4]

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 plates from each replicate dilution) and multiply by the dilution factor to calculate (cfu/mL) microbial population of both the control blank and test system.

[2] 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.)

[3] *U.S. Pharmacopoeia, 24th Revision*, The United States Pharmacopoeia Convention, Inc. Rockville, MD, 2000.

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

9. Basic Procedure

9.1 Select the test concentrations of the test material. The concentrations selected may reflect the anticipated concentration of the test material during use. Each concentration is tested at least in duplicate. Each recovery sample is plated in duplicate.

9.2 Prepare each test concentration at least in duplicate. Dilutions should be prepared using sterile distilled water. Dilution using other materials, such as saline or a buffer may be appropriate if test material is typically diluted that way under conditions of use or for informational purposes. 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 room temperature; 30 ± 2°C temperature of human skin; and 38 ± 2°C temperature of "warm" water). A solid test material(s) 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 where 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 reproducibly conduct the test sampling in this short 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 over a period of time in order to construct a kinetic kill model. Additional contact times may be selected at the discretion of the investigator.

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

9.5 Start mixing the test sample, add the appropriate inoculum suspension to the sample or control blank and maintain mixing to disperse the inoculum suspension. The inoculum suspension should be uniformly mixed. Maintaining uniform mixing throughout the test is crucial for test repeatability. Where applicable mix carefully to minimize foam formation. The formation of foam may cause anomalous results.

NOTE 4—Methods of mixing include use of a vortex, stomacher, syringe, positive displacement pipette, constant velocity mix plate or other methods that rapidly disperse inoculum into the test material, while maintaining uniform mixing of the test system. Viscous materials may cause difficulty in mixing sample from the sample tube.

9.6 At predetermined time intervals remove an aliquot, typically 1 mL, from the sample/inoculum container 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 Fig. 1.

9.7 Recover viable organisms from appropriate dilutions by culturing in duplicate (see 7.3) by use of 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 for growth of surviving organisms, without overgrowth of colonies, making enumeration difficult.

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

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

Step 1: Transform the measured initial microbial population of the control blank (see 8.2.4.1) into the $\log_{10}$ scale.

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

Step 3: Transform the measured surviving microbial population (see 9.9) into the $\log_{10}$ scale.

Step 4: If more than one tube was used for determining the surviving microbial population, transform each measured value to the $\log_{10}$ scale first, then calculate the mean and variance for associated with the use of multiple tubes.

Step 5: Calculate the log reduction.

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

Step 6: Calculate the standard error of the mean

$$\text{Standard error} =$$
$$\text{square root [(variance of the log}_{10}\text{ measured initial microbial population/}$$
$$\text{number of replicates) +}$$
$$\text{(variance of the log}_{10}\text{ surviving microbial population/number of replicates)]}$$

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

9.11 *Formula for Calculating Percent Reduction*:

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

10. Report

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

10.1.1 *Test Organism*—Specify the genus, species, origin, growth temperature, transfer frequency and incubation period. Describe the rationale for selection of the test organisms.

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

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

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

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

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

10.1.7 *Neutralization*—Specify neutralization technique employed. Show data demonstrating neutralization (see 3.1).

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

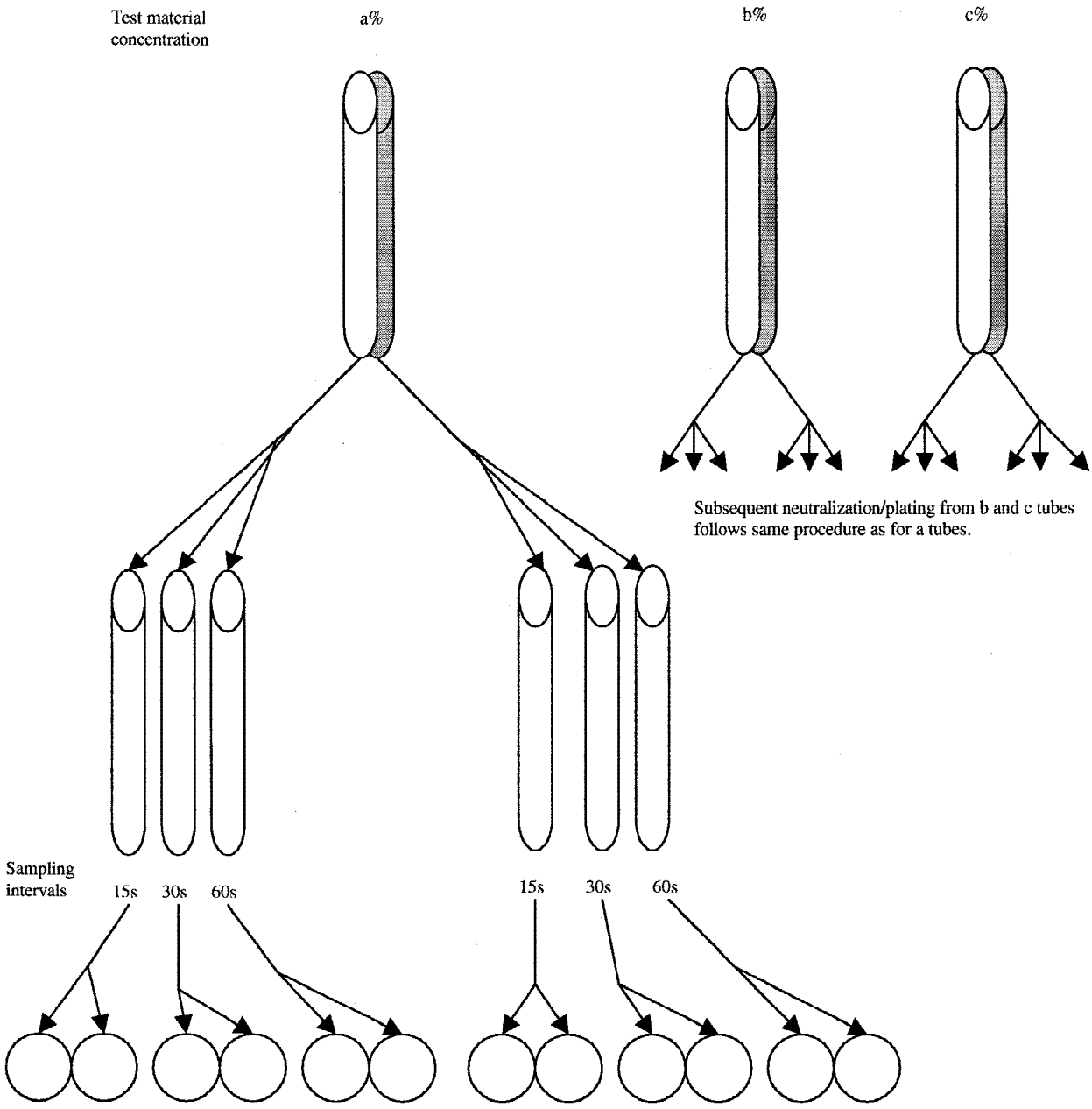

FIG. 1 Time Kill Dilution/Plating Scheme

11. Precision and Bias

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

11.2 *Bias*—The bias of this practice will be affected by the same variables listed in 11.1. Two additional variables depend on the specific procedures used to evaluate a test material:

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

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

12. Keywords

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

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