



Standard Test Method for Assessment of Antimicrobial Activity for Water Miscible Compounds Using a Time-Kill Procedure¹

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1. Scope

1.1 This test method measures the changes of a population of aerobic and anaerobic microorganisms within a specific sampling time when tested against antimicrobial test materials *in vitro*. The organisms used are standardized as to growth requirements and inoculum preparation and must grow under the conditions of the test. The primary purpose of this test method is to provide a set of standardized conditions and test organisms to facilitate comparative assessments of antimicrobial materials miscible in aqueous systems.

1.2 This test method allows the option of using a test sample size of 10 mL or 100 mL.

1.3 Knowledge of microbiological techniques is required for this procedure.

1.4 Aseptic technique should be practiced at all times.

1.5 In this test method, SI units are used for all applications, except for distance in which case inches are used and SI units follow in parentheses.

1.6 *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 limitations prior to use.*

2. Referenced Documents

2.1 ASTM Standards:²

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

¹ This test method 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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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.

3. Terminology

3.1 Definitions:

3.1.1 *antimicrobial, n*—describes an agent that kills or inactivates microorganisms or suppresses their growth or reproduction.

3.1.2 *drug, n*—articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals. Drugs are intended to affect the structure or any function of the body of man or other animals.

3.1.3 *initial microbial population, n*—bacterial count (CFU/mL) in the final volume of test material. Also known as initial bacterial population, numbers control or control.

3.1.4 *inoculum, n*—the viable microorganisms used to contaminate a sample, device or surface, often expressed as to number and type.

3.1.5 *microbiocide, n*—a physical or chemical agent that kills microorganisms.

3.1.6 *neutralization, n*—the process for inactivating or quenching the activity of a microbiocide. Often achieved through chemical or physical means (for example, filtration or dilution).

3.1.7 *room temperature, n*—temperature in the range of 20 to 30°C (68 to 85°F).

3.1.8 *test material, n*—a formulation which incorporates antimicrobial ingredient(s). Also known as test formulation.

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

4. Summary of Test Method

4.1 A dilution/aliquot of the test material is brought into contact with a known population of test organisms for specified periods of time, at a specified temperature. The activity of the test material is quenched at specified sampling intervals (example 15, 30, and 60 s, or any range covering several minutes or hours) with an appropriate neutralizing technique. The test material is neutralized at the sampling time and the surviving microorganisms enumerated. The percent and log₁₀ reduction, from an initial microbial population 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 *Adjustable or Fixed Volume Pipet*—Capable of dispensing 0.1 mL and 1.0 mL.

6.2 *Anaerobic Jar or Incubator*—Any incubator or apparatus in an incubator that creates an environment having a level of oxygen that does not support the growth of oxygen-requiring microorganisms. Required only for organisms that need an anaerobic environment to grow.

6.3 *Balance*—Any suitable laboratory balance with a minimum readability of 0.01 g.

6.4 *Beakers and Magnetic Stir Bars*—For 100 mL sample size. A 250 mL beaker containing a 51 by 8 ± 2 mm magnetic stir bar. The beaker and stir bar should be sterile.

6.5 *Colony Counter*—Any of several types may be used.

6.6 *Incubator*—Any incubator capable of maintaining a suitable temperature $\pm 2^\circ\text{C}$ may be used.

6.7 *Laboratory Centrifuge*—Any centrifuge capable of producing 3200 r/min (1520 RCF).

6.8 *Magnetic Stirring Plate*—Any rotor powered magnetic stirrer.

6.9 *Positive Displacement Pipet*—1.0 mL capacity. Required for viscous test materials.

6.10 *Sterilizer*—Any suitable steam sterilizer capable of producing the conditions of sterility.

6.11 *Sterile Container*—Any container of sufficient size to dilute test material into.

6.12 *Test Tubes with Cap*—Sterile. Alternate sample container to (7.5) for 10 mL sample size. Size of test tube should allow the thorough mixing of samples.

6.13 *Timer (Stop Clock)*—One that displays minutes and seconds.

6.14 *Vortex Mixer*—Any suitable vortex mixer capable of mixing test material and diluents.

6.15 *Waterbath*—Any waterbath capable of maintaining suitable temperature.

7. Reagents and Materials

7.1 *Bacteriological Loops*—Any type of disposable sterile or sterilizable bacteriological loop is suitable.

7.2 *Bacteriological Pipets*—Sterile. 1.1, 2.0, 5.0, 10.0 mL capacity.

NOTE 1—Pre-sterilized/disposable bacteriological pipets are available from most local laboratory supply houses.

7.3 *Broth Growth Medium*—Sterile soybean-casein digest broth (tryptic soy broth) or other broth media appropriate to support growth of the test organisms.

7.4 *Dilution Fluid or Diluent*—Sterile Butterfield's buffered phosphate diluent³ or other suitable diluent with appropriately validated neutralizers. Perform Test Method E1054 to determine what diluent or neutralizers are required. Volume is 9.0 mL after sterilization.

7.5 *Flip Top Centrifuge Tubes*—Sterile. For 10 mL sample size. Minimum of 50 mL capacity.

NOTE 2—Pre-sterilized/ disposable flip-top centrifuge tubes are available from most laboratory supply houses.

7.6 *Petri Dishes*—100 by 15 mm. Required to plate samples and control.

NOTE 3—Pre-sterilized/disposable plastic petri dishes are available from most local laboratory supply houses.

7.7 *Physiological Saline*—Sterile. Used to prepare inoculum.

7.8 *Pipet Tips*—Sterile. 0.1 and 1.0 mL capacity.

7.9 *Positive Displacement Pipet Tips*—Sterile. 1.0 mL capacity.

7.10 *Solid Growth and Plating Medium*—Sterile soybean-casein digest agar (tryptic soy agar) or other solid media appropriate to support growth of the test organism(s) with appropriately validated neutralizers. Perform Test Method E1054 to determine what neutralizers are required. Should be tempered to between 40 to 50°C if using a pour plate method.

7.11 *Water*—Sterile deionized or distilled water.

8. Hazards

8.1 All test organisms listed for use in this method fall under the Biosafety level 1 or 2 categories and should be handled in accordance with CDC and NIH guidelines.⁴

9. Calibration and Standardization

9.1 Ensure that all equipment used in this test method has been calibrated and standardized as required for that piece of equipment.

10. Test Organisms

10.1 The following list of organisms may be used in this procedure. This list is not all inclusive and other organisms may be used. 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 the test conditions.

10.2 The organisms listed shall be maintained as specified by ATCC or other validated methods.

10.2.1 *Acinetobacter* species.

10.2.2 *Candida albicans* ATCC 10231.

10.2.3 *Enterobacter* species.

10.2.4 *Enterococcus faecalis* ATCC 29212.

10.2.5 *Enterococcus faecium*.

³ Horowitz, W., (Ed.), *Official Methods of Analysis of the AOAC*, 17th Ed., Sec. 6.3.03 A.(f), Chapter 6, 2000, p.10. Official Methods of Analysis of AOAC International, Gaithersburg, MD.

⁴ Richmond, J. Y. and McKinney, R. W. (eds.), 1999, *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed., Washington DC, U.S. Government Printing Office.