Designation: E 1054 – 02

Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents[1]

This standard is issued under the fixed designation E 1054; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 These methods are used to determine the effectiveness of procedures and agents for inactivating (neutralizing, quenching) the microbiocidal properties of antimicrobial agents and to ensure that no components of the neutralizing procedures and agents, themselves, exert an inhibitory effect on microorganisms targeted for recovery.

NOTE 1—Knowledge of microbiological and statistical techniques is required for these procedures. These methods are not applicable to testing with viruses (see Test Method E 1482).

1.2 *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]

E 645 Test Method for Effectiveness of Microbicides Used in Cooling Systems

E 1115 Test Method for Evaluation of Surgical Hand Scrub Formulations

E 1482 Test Method for Neutralization of Virucidal Agents in Virucidal Effectiveness Evaluations

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *antimicrobial agent*—a test formulation, chemical compound, or product designed to prevent the growth of microbes either by inhibiting growth or destroying the microbe.

3.1.2 *antimicrobial effectiveness evaluation*—a determination of microbiocidal properties of an antimicrobial agent by methods, such as Test Methods E 645 and E 1115.

3.1.3 *CFU/mL*—colony-forming units of a microorganism per millilitre of fluid.

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

3.1.5 *neutralizer effectiveness*—a neutralizer's ability to inactivate, neutralize, or quench the microbiocidal properties of an antimicrobial agent.

3.1.6 *neutralizer toxicity*—any inhibitory effects a neutralizer may have on the survival of a microbial population.

3.1.7 *test material control*—an evaluation of the activity of an test material in reducing a known population of microorganisms.

3.1.8 *test organism viability*—the population or viability of a challenge microorganism used in a neutralization assay.

4. Summary of Test Methods

NOTE 2—The neutralization test method selected must be identical to the method used in the antimicrobial effectiveness evaluation.

4.1 *Neutralization Assay with Recovery on Solid Medium*—Neutralization assay for antimicrobial effectiveness tests that recover and quantify microorganism populations on solid (agar) media. This method is appropriate for antimicrobial agents that can be chemically inactivated or diluted to sub-inhibitory levels.

4.2 *Neutralization Assay with Recovery in Liquid Medium*—Neutralization assay for antimicrobial effectiveness tests that recover surviving microorganism populations in liquid media for a growth/no growth determination. This method is appropriate for antimicrobial agents that can be chemically inactivated or diluted to sub-inhibitory levels.

4.3 *Neutralization Assay with Recovery by Membrane Filtration*—Neutralization assay for antimicrobial effectiveness tests that recover and quantify microorganism populations by using membrane filtration. This method is appropriate for antimicrobial agents that cannot be chemically inactivated or diluted to sub-inhibitory levels. This method should not be used when difficulties are incurred during the filtration process.

[1] These test methods are under the jurisdiction of ASTM Committee E35 on Pesticides and are 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.

5. Significance and Use

5.1 The effectiveness of antimicrobial agents such as disinfectants, sanitizers and antiseptics are measured by their ability to kill microorganisms at or for a specified contact time. Accurate determination of antimicrobial effectiveness therefore requires efficient and effective inactivation (neutralization) of the antimicrobial agent. Inefficient or incomplete neutralization will permit killing or inactivation of microorganisms to continue beyond the experimental exposure time, resulting in an over-estimation of antimicrobial activity.

5.2 The neutralization methods commonly used in antimicrobial effectiveness evaluations are chemical inactivation, dilution and filtration. All critical parameters, for example, media, microorganism(s), equipment, and temperature of solutions, of the antimicrobial effectiveness evaluation must be mimicked when evaluating a neutralization procedure to be used in the antimicrobial effectiveness evaluation.

5.3 The evaluation must include at least three replications (five replications in Section 9) so that a statistical analysis can be performed with the recovery data. The number of replicates used in the evaluation depends on the statistical significance required for the expected results, the variability encountered in the evaluation, and the relative efficacy of the neutralization procedure.

5.4 A limitation of these evaluation procedures is that they use microorganisms that have not been exposed to an antimicrobial. Under experimental conditions, cells exposed to neutralization procedures are likely to be damaged to different degrees by the antimicrobial agent. Sublethal injury may be a factor in recovery and the role of the neutralization procedure in recovery of injured organisms should be examined.

NOTE 3—Ideally, all microorganisms used in the antimicrobial effectiveness evaluation should be tested in the neutralization assay. However, "representative" organisms may be selected for testing, as judged appropriate by the investigator. The investigator is cautioned that failure to identify neutralizer efficacy and toxicity for all microorganisms could result in exaggerated microbial reductions in an antimicrobial effectiveness evaluation. Also, for studies involving multiple antimicrobial products and a sample containing multiple species of microorganisms (for example, skin flora), a single neutralizing procedure and/or combination of agents suitable for the multiple products must be used for testing.

6. Apparatus

6.1 Standard bacteriological devices and equipment should be used for performance of the neutralization assay.

6.2 *Colony Counter*—Any of several types may be used; for example, Quebec colony counters and similar devices, or automated, computerized plater/counter systems.

6.3 *Incubator*—Any incubator capable of maintaining an appropriate temperature for growth of the microorganism may be used.

6.4 *Sterilizer*—Any steam sterilizer capable of producing the conditions of sterilization.

6.5 *Timer (stopwatch)*—One that displays hours, minutes, and seconds.

6.6 *Vortex Mixer or equivalent*.

6.7 *Membrane Filter Units*—Any sterilizable unit that permits filtration of microorganisms for enumeration. The membrane filter unit should be suitable for testing the antimicrobial agent and recovery of the microorganisms.

7. Reagents and Materials

7.1 *Phosphate Buffered Saline Dilution Water*—PBS (see Test Method E 645).

7.1.1 *Phosphate Buffer Solution, Stock*—Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust pH to 7.2 ± 0.2 with 0.1 N NaOH or 0.1 N HCl and bring to 1000 mL with deionized water.

7.1.2 *Phosphate Buffer Saline Dilution Water*—Add 1.25 mL of stock phosphate buffer solution and 8.75 g of NaCl to a volumetric flask, fill with deionized water to the 1000 mL mark, and mix. Final pH should be 7.2 ± 0.2. Sterilize by filtration or autoclave.

7.2 Because the types of materials and reagents required for various antimicrobial effectiveness evaluations are so diverse, it is impractical to list them in this method. The specific materials and reagents to be used in the antimicrobial effectiveness evaluation, however, should be tested in the neutralization assay to confirm that the antimicrobial agent is being neutralized in a particular evaluation.

7.3 Table 1 provides a partial list of materials that have been employed by researchers to inactivate the microbiocidal properties of various antimicrobial agents. This list is provided as a guide for selecting neutralizers. A neutralization assay should be performed to determine a selected neutralizer's effectiveness.

8. Neutralization Assay with Recovery on Solid Medium (Fig. 1)

8.1 At least three replicates are required for these procedures. The number of replicates used in the evaluation depends on the statistical significance required for the expected results, the variability encountered in the evaluation, and the relative efficacy of the neutralization procedure.

8.2 All tests must be performed in a timely manner so that replication of the test organism does not occur.

8.3 *Test A—Neutralizer Effectiveness*:

8.3.1 Add a volume of product, or solution containing product, to neutralizer that will result in the same dilution ratio to be used in the antimicrobial effectiveness evaluation. If the antimicrobial effectiveness evaluation will employ the use of carriers, use instead a carrier containing an amount of product representative of that to be used in the test.

NOTE 4—The dilution ratio of product to neutralizer can be manipulated to determine the dilution at which adequate neutralization of the product will occur, particularly when testing products not readily neutralized by chemical means.

NOTE 5—The sequence of product-into-neutralizer, followed by the challenge microorganism, allows the neutralizing action to take place. If the microorganism is introduced into the neutralizing solution prior to adding the product, there is possibility of the product acting on the microorganism there by reducing the population and disqualifying the neutralizer.

8.3.2 Within 5 s of execution of 8.3.1, inoculate the product/neutralizer mixture with a volume of the challenge microorganism suspension so that the resulting suspension contains 30 to 100 CFU/mL of the microorganism.

TABLE 1 Processes Applied for Neutralization of Certain Antimicrobial Agent[A]

Antimicrobial Agent	Neutralizers/Inactivators
Alcohols	
Isopropanol, Phenoxyethanol	Polysorbate 80, dilution to sub-inhibitory levels
Aldehydes	
2-Bromo-2-nitroproprane-1, 3-diol (bronopol)	Serum, cysteine, thiosulfate, thioglycolate, metabisulfite
Formaldehyde	Sodium sulfite, ammonia, histamine
Glutaraldehyde	Dilution to sub-inhibitory levels, sodium bisulfite, sodium sulfite, glycine, cystine, cysteine
Chlorallytriazaazoniaadamantane (Dowicil 200)	Dilution to sub-inhibitory levels
Dimethyloldimethyl hydantoin (Glydant)	Dilution to sub-inhibitory levels
Biguanides and Bis-biquanides	
Chlorhexidine	Lecithin/polysorbate 80, sodium oleate
Polyhexamethylene biguanide HCL (Cosmocil CQ)	Polysorbate 80/lecithin
Phenolics	
Phenylphenol, Chloroxylenol, Cresols, Chlorocresols, Phenol	Nonionic surfactants, polysorbate 80, and/or dilution to sub-inhibitory levels
Bis-Phenols	
Triclosan	>10 % polysorbate 80/lecithin, and dilution to sub-inhibitory levels
Hexachlorophene	>10 % polysorbate 80/lecithin, and dilution to sub-inhibitory levels
Quaternary Ammonium Compounds	
Cetrimide, Benzalkonium and Benzethonium Chloride	Lecithin/polysorbate, suramin sodium, organic material, 0.5 % polysorbate 80, cyclodextrins
Mercurials	Sulfhydryl compounds, thioglycolic acid, thiosulfate, bisulfite, ammonium sulfite
Organic Acids	
Benzoic, Propionic, Sorbic	Nonionic surfactants, dilution to sub-inhibitory levels, pH 7 of above
Halogens	
Hypochlorite	Thiosulfate and/or dilution to sub-inhibitory levels
Iodine	Thiosulfate, polysorbate 80, skim milk
Bromine	Thiosulfate and/or dilution to sub-inhibitory levels
EDTA	Mg^{+2} or Ca^{+2} ions
Imidazolidinyl urea	Dilution to sub-inhibitory levels
Methyl-, and methylcholoroisothiazolinone (Kathon)	Amines, sulfites, mercaptans, sodium bisulfite, heparin
Parabens	
Methyl-, ethyl-, propyl-, butyl-parahydroxybenzoate	Lecithin, filtration, dilution to sub-inhibitory levels, polysorbate surfactants, 1 % polysorbate 80 or 20
Hydrogen Peroxide	Catalase
Peroxyacetic Acid	Sodium Thiosulfate

[A] Sutton, S. V. W., "Neutralizer Evaluations as Control Experiments for Antimicrobial Effectiveness Tests," Ch. 3 in *Handbook of Disinfectants and Antiseptics*, Marcel-Dekker, NY, 1996, p. 300.

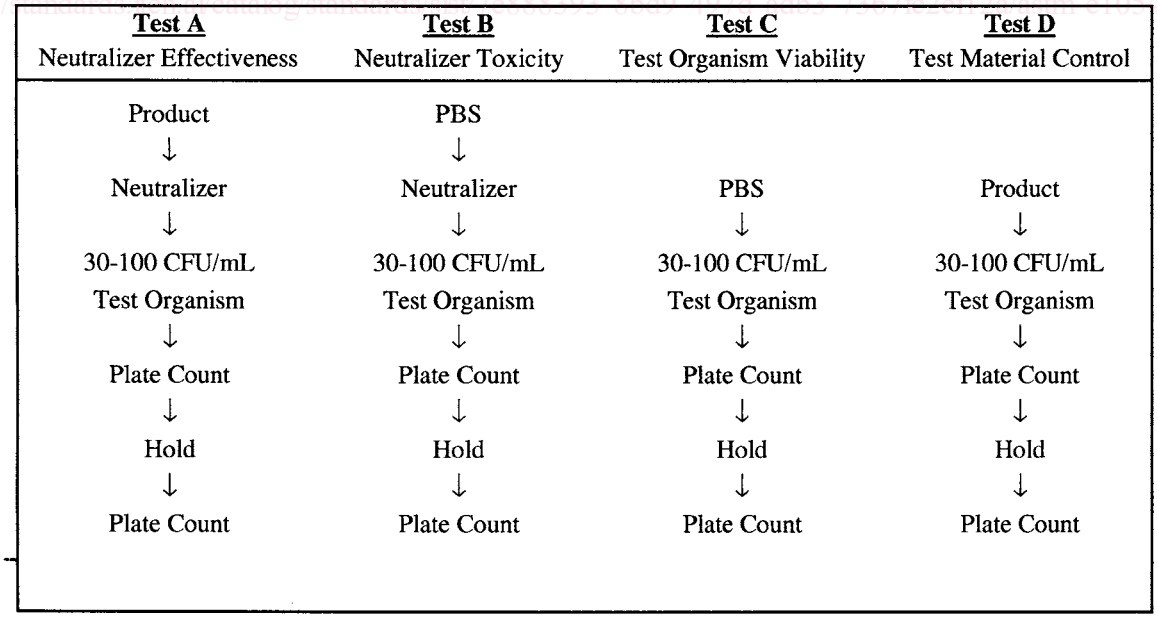

FIG. 1 Testing Schema for Neutralization Assay with Recovery on Solid Medium

NOTE 6—The challenge inoculum should be prepared in the same manner used in the antimicrobial effectiveness evaluation. The volume of

the challenge inoculum should be kept to a minimum so it does not cause significant dilution of the product/neutralizer mixture.

8.3.3 Within 1 min of execution of 8.3.2, enumerate the product/neutralizer/microorganism suspension by quantitative pour or spread plates, in duplicate, using appropriate plating medium. If neutralizers are to be incorporated in the plating medium for the antimicrobial effectiveness evaluation, use this same medium for plating the suspension.

8.3.4 Allow the product/neutralizer/microorganism suspension to stand for the longest exposure period representative of that to be used in the antimicrobial effectiveness evaluation. For example, if the product/neutralizer/microorganism from the antimicrobial effectiveness evaluation will be plated within 30 min, then the longest exposure period for the neutralization assay is 30 min.

8.3.5 After the hold-time, enumerate the product/neutralizer/microorganism suspension by quantitative pour or spread plates, in duplicate, using an appropriate plating medium. If neutralizers are to be incorporated in the plating medium for the antimicrobial effectiveness evaluation, use this same medium for plating the suspension.

NOTE 7—The duration of the hold time must not be such that replication of the test organism introduces a variable.

8.3.6 Repeat this procedure (8.3.1-8.3.5) an additional two times, for a total of three replicates.

8.3.7 Incubate the plates under the same conditions as those to be used in the antimicrobial effectiveness evaluation.

8.4 *Test B—Neutralizer Toxicity*:

8.4.1 Add a volume of PBS or other appropriate buffering agent to neutralizer that will result in the same dilution ratio as that used in Test A (see 8.3.1).

8.4.2 Within 5 s of execution of 8.4.1, inoculate the PBS/neutralizer mixture with a volume of the challenge microorganism suspension so that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see Note 6).

8.4.3 Within 1 min of execution of 8.4.2, enumerate the PBS/neutralizer/microorganism suspension by quantitative pour or spread plates, in duplicate, using an appropriate plating medium. If neutralizers are to be incorporated in the plating medium for the antimicrobial effectiveness evaluation, use this same medium for plating the suspension.

8.4.4 Allow the PBS/neutralizer/microorganism suspension to stand for the same period used in Test A (see 8.3.4).

8.4.5 After the hold-time, enumerate the PBS/neutralizer/microorganism suspension by quantitative pour or spread plates, in duplicate, using an appropriate plating medium. If neutralizers are to be incorporated in the plating medium for the antimicrobial effectiveness evaluation, use this same medium for plating the suspension.

8.4.6 Repeat this procedure (8.4.1-8.4.5) an additional two times, for a total of three replicates.

8.4.7 Incubate the plates under the same conditions as those to be used in the antimicrobial effectiveness evaluation.

8.5 *Test C—Test Organism Viability*:

8.5.1 Inoculate a volume of PBS or other appropriate buffering agent with a volume of the challenge microorganism suspension so that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see Note 6).

8.5.2 Within 1 min of execution of 8.5.1, enumerate the PBS/microorganism suspension by quantitative pour or spread plates, in duplicate, using an appropriate plating medium that does not contain neutralizers and is not a selective plating medium.

8.5.3 Allow the PBS/microorganism suspension to stand for the same exposure period used in Test A (see 8.3.4).

8.5.4 After the hold-time, enumerate the PBS/microorganism suspension by quantitative pour or spread plates, in duplicate, using an appropriate plating medium that does not contain neutralizers and is not a selective plating medium.

8.5.5 Repeat this procedure (8.5.1-8.5.4) an additional two times, for a total of three replicates.

8.5.6 Incubate the plates under the same conditions as those to be used in the antimicrobial effectiveness evaluation.

8.6 *Test D—Test Material Control*:

NOTE 8—A test of a product's antimicrobial effectiveness is required to demonstrate that the neutralizer actually did neutralize the activity of an antimicrobial agent.

8.6.1 Inoculate the product with a volume of the challenge microorganism suspension so that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see Note 6).

8.6.2 Hold the product/microorganism suspension for an exposure period necessary to allow detection of an antimicrobial effect. The hold time must not be longer than the hold time in Test A (see 8.3.4).

8.6.3 After the hold time, enumerate the product/microorganism suspension by quantitative pour or spread plates, in duplicate, using an appropriate plating medium that does not contain neutralizers.

8.6.4 Repeat this procedure (8.6.1 and 8.6.2) an additional two times, for a total of three replicates.

8.6.5 Incubate the plates under the same conditions as those to be used in the antimicrobial effectiveness evaluation.

9. Neutralization Assay with Recovery in Liquid Medium (Fig. 2)

9.1 At least five replicates are required for these procedures. The number of replicates used in the evaluation depends on the statistical significance required for the expected results, the variability encountered in the evaluation, and the relative efficacy of the neutralization procedure.

9.2 All tests must be performed in a timely manner so that replication of the test organism does not occur.

9.3 *Test A—Neutralizer Effectiveness*:

9.3.1 Add a volume of product or solution containing product to neutralizer/nutrient medium that will result in the same dilution ratio to be used in the antimicrobial effectiveness evaluation (see Note 4). If the antimicrobial effectiveness evaluation will employ the use of carriers, use instead a carrier containing an amount of product representative of that to be used in the test.

9.3.2 Within 5 s of execution of 9.3.1, inoculate the product/neutralizer/nutrient medium mixture with a volume of the challenge microorganism suspension so that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see Note 6).