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Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents¹

This standard is issued under the fixed designation E1054; 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 reappraisal. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reappraisal.

1. Scope

1.1 These test methods are used to determine the effectiveness of procedures and agents for inactivating (neutralizing, quenching) the microbicidal 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 suitable when testing the virucidal activity of microbicides (see Test Method E1482).

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.3 *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

E645 Test Method for Efficacy of Microbicides Used in Cooling Water Systems

E1115 Test Method for Evaluation of Surgical Hand Scrub Formulations

E1482 Test Method for Neutralization of Virucidal Agents in Virucidal Efficacy Evaluations

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

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

¹ These test methods are under the jurisdiction of ASTM Committee E35 on Pesticides and Alternative Control Agents 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.

3.1.2 *antimicrobial effectiveness evaluation, adj/n*—a determination of microbicidal properties of an antimicrobial agent by methods, such as Test Methods E645 and E1115.

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

3.1.4 *neutralization, n*—a physical or chemical procedure that inactivates or quenches the microbicidal properties of an antimicrobial agent.

3.1.5 *neutralizer effectiveness, adj/n*—ability of a neutralization procedure to inactivate or quench the microbicidal properties of an antimicrobial agent.

3.1.6 *neutralizer toxicity, adj/n*—any inhibitory effects a neutralization procedure may have on the survival of a microbial population.

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

3.1.8 *test organism viability, adj/n*—the population of a challenge microorganism used in a neutralization assay.

3.1.9 *viability, n*—the ability of a challenge microorganism to form colonies or grow on a nutrient medium.

3.1.9.1 *Discussion*—In the context of these test methods, “viability” is understood to be synonymous with culturability.

4. Summary of Test Methods

NOTE 2—The neutralization test method selected must be consistent with the methods of testing used in the antimicrobial effectiveness evaluation.

4.1 *Neutralization Assay with Recovery on Semi-solid Medium*—Neutralization assay for antimicrobial effectiveness tests that recover and quantify microbial populations on solid (agar) media. This method is appropriate for antimicrobial agents that are chemically inactivated or diluted to sub-inhibitory levels and performed entirely *in vitro* or including an *in vivo* component to verify neutralization of an antimicrobial formulation sampled from the skin of a human volunteer.

4.2 *Neutralization Assay with Recovery in Liquid Medium*—Neutralization assay for antimicrobial effectiveness tests that recover surviving microbial populations in liquid media for a growth/no growth determination. This method is appropriate for antimicrobial agents that are 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 microbial populations by using membrane filtration. This method is appropriate for antimicrobial agents that cannot be chemically inactivated or diluted to sub-inhibitory levels, as well as for those that can be.

5. Significance and Use

5.1 The effectiveness of antimicrobial agents incorporated into disinfectants, sanitizers, and antiseptics is measured by their ability to kill microorganisms within a specified contact time. Hence, accurate determination of antimicrobial effectiveness requires complete and immediate 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 overestimation of antimicrobial activity.

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

5.3 The neutralization evaluation must include at least three replications (five replications in Section 9) so that a statistical analysis of the recovery data can be performed. The number of replicates used in the evaluation depends on the statistical significance required for the expected results, the variability encountered in the data, and the relative effectiveness 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 agent. 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. This method is not intended to assess injured organism recovery.

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 a study testing multiple antimicrobial formulations, and in which samples will contain multiple species of microorganisms (for example, skin flora) that are exposed to the formulations, a single procedure and/or combination of agents suitable for neutralizing the antimicrobial activities of the multiple formulations 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 test 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 must be chemically compatible with the antimicrobial agent and appropriate to efficient recovery of the test microorganisms.

7. Reagents and Materials

7.1 *Phosphate Buffered Saline Dilution Water*—PBS (see Test Method E645).

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 adjusted to 7.2 ± 0.2 , if necessary. 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 list of materials employed by researchers to inactivate the microbicidal properties of various antimicrobial agents. This list is provided as a guide for selecting neutralizers and is not exhaustive. A neutralization assay must be performed to determine a selected neutralizer's effectiveness.

8. Neutralization Assay with Recovery on Semi-solid Medium (Fig. 1)

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

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

8.3 *Test A—Neutralization Effectiveness:*

8.3.1 Inoculate the neutralizer with a volume of the challenge microbial suspension to result in a suspension that contains 30 to 100 CFU/mL of the microorganism.

NOTE 4—The challenge inoculum should be prepared in the same manner to be used in the antimicrobial effectiveness evaluation. The

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-nitropropane-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
N-(3-Chloroallyl)hexaminium Chloride (Dowicide Q)	Dilution to sub-inhibitory levels
Dimethylol-5, 5-dimethylhydantoin (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 and Silver	Sulfhydryl compounds, thioglycolic acid, thiosulfate, bisulfite, ammonium sulfite
Organic Acids Benzoic, Propionic, Sorbic	Nonionic surfactants, dilution to sub-inhibitory levels, pH 7 or 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 dimethylchloroisothiazolinone (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.

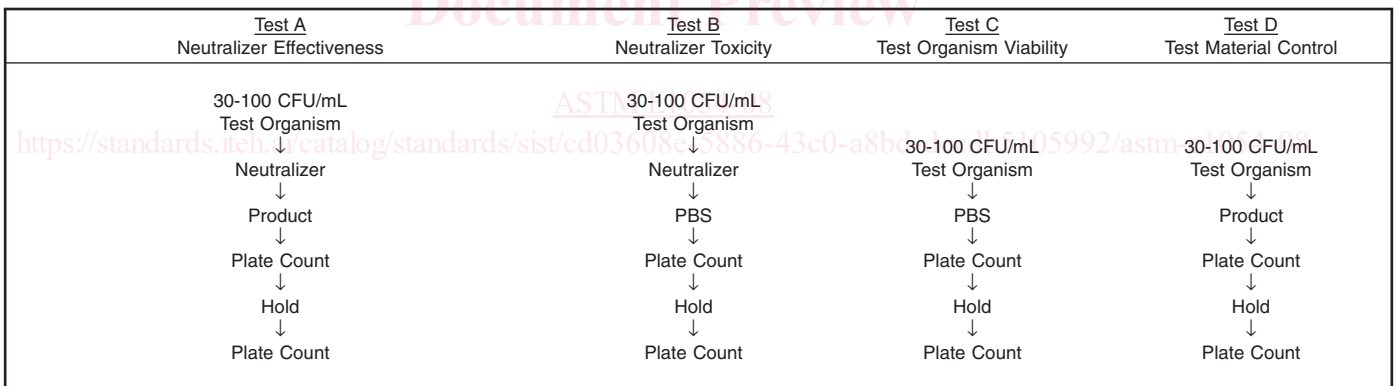


FIG. 1 Testing Schema for Neutralization Assay with Recovery on Semi-solid Medium

volume of the challenge inoculum should be kept to a minimum so that it does not cause significant dilution.

8.3.2 Add a volume of product, or solution containing product, to the neutralizer/microbial suspension that will result in the same dilution ratio used in the antimicrobial effectiveness evaluation. If the antimicrobial effectiveness evaluation will employ the use of carriers, use instead a carrier bearing an amount of product used in the effectiveness evaluation.

NOTE 5—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.

8.3.3 Within 1 min of execution of 8.3.2, transfer aliquots of the product/neutralizer/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium. If neutralizers are 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/microbial suspension to stand for the longest exposure period representative of that 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, transfer aliquots of the product/neutralizer/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium. If neutralizers are incorporated in the plating medium for the antimicrobial effectiveness evaluation, use this same medium for plating the suspension.

NOTE 6—The duration of the hold time must not be such that proliferation of the test organism introduces a variable.

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

8.3.7 Incubate the plates under the same conditions as those used in the antimicrobial effectiveness evaluation, and following incubation, enumerate colony-forming units.

8.4 Test B—Neutralizer Toxicity:

8.4.1 Inoculate the neutralizer with a volume of the challenge microbial suspension such that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see **Note 4**).

8.4.2 Add a volume of sterile PBS or other appropriate buffering agent to the neutralizer/microbial suspension that will result in the same dilution ratio as that used in Test A (see 8.3.2).

8.4.3 Within 1 min of execution of 8.4.2, transfer aliquots of the PBS/neutralizer/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium. If neutralizers are 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/microbial suspension to stand for the same period used in Test A (see 8.3.4).

8.4.5 After the hold-time, transfer aliquots of the PBS/neutralizer/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium. If neutralizers are 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 or more times, for a total of at least three replicates.

8.4.7 Incubate the plates under the same conditions as those used in the antimicrobial effectiveness evaluation, and following incubation, enumerate the colony-forming units.

8.5 Test C—Test Organism Viability:

8.5.1 Inoculate a volume of sterile PBS or other appropriate buffering agent with a volume of the challenge microbial suspension such that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see **Note 4**).

8.5.2 Within 1 min of execution of 8.5.1, transfer aliquots of the PBS/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium that does not contain neutralizers and is not a selective or differential medium.

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

8.5.4 After the hold-time, transfer aliquots of the PBS/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium that does not contain neutralizers and is not a selective or differential medium.

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

8.5.6 Incubate the plates under the same conditions as those used in the antimicrobial effectiveness evaluation, and following incubation, enumerate the colony-forming units.

8.6 Test D—Test Material Control:

NOTE 7—A test of a product's antimicrobial effectiveness is necessary to demonstrate that the neutralizer actually does neutralize the activity of an antimicrobial agent. However, performance of the Test Material Control phase is situational and may not be necessary for specific formulations with which the researcher has prior experience.

8.6.1 Inoculate the product with a volume of the challenge microbial suspension such that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see **Note 4**).

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

8.6.3 After the hold time, transfer aliquots of the product/microbial suspension to pour or spread plates, in duplicate, using an appropriate semi-solid medium that does not contain neutralizers and is not a selective or differential medium.

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

8.6.5 Incubate the plates under the same conditions as those used in the antimicrobial effectiveness evaluation, and following incubation, enumerate the colony-forming units.

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 data, and the relative effectiveness of the neutralization procedure. The same nutrient medium should be used in all phases of the assay.

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

9.3 Test A—Neutralization Effectiveness:

9.3.1 Inoculate the neutralizer-nutrient medium with a volume of the challenge microbial suspension such that the resulting suspension contains 30 to 100 CFU/mL of the microorganism (see **Note 4**).