Designation: E1054 – 22

Standard Practices for Evaluation of Inactivators of Antimicrobial Agents[1]

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 reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 These test procedures are used to determine the effectiveness of methodologies procedures and materials intended for inactivating (neutralizing, quenching) the microbicidal properties of antimicrobial agents; to ensure that no components of the neutralizing procedures and materials, themselves, exert an inhibitory effect on microorganisms targeted for recovery; and to demonstrate that the antimicrobial chemistry tested is microbicidal.

1.2 Knowledge of microbiological and statistical techniques is required for these procedures.

NOTE 1—These methods are not suitable when testing the virucidal activity of microbicides (see Test Method E1482).

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E645 Practice for Evaluation of Microbicides Used in Cooling Water Systems

E1115 Test Method for Evaluation of Surgical Hand Scrub Formulations

E1482 Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in these practices, refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *antimicrobial effectiveness evaluation, n*—a determination of microbicidal properties of an antimicrobial agent by methods, such as Practice E645 and Test Method E1115.

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

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

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

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

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

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

3.2.7.1 *Discussion*—In the context of these test methods, "viability" is understood to be synonymous with cultivability.

4. Summary of Practices

NOTE 2—The neutralization test procedure 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 semi-solid (agar) media. This method is appropriate for antimicrobial agents that are chemically inactivated or diluted to

[1] These practices are under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and are the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

Current edition approved Oct. 1, 2022. Published October 2022. Originally approved in 1985. Last previous edition approved in 2021 as E1054 – 21$^{\varepsilon 1}$. DOI: 10.1520/E1054-22.

[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.

sub-inhibitory levels and performed entirely *in vitro* or including an *in vivo* component, such as 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 in the performance of selected neutralization procedure.

5.3 The neutralization evaluation must include at least three replications (five replications in Section 9) so that a statistical analysis of the microbial 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 effect of the neutralization procedure on recovery of injured organisms should be examined. This method is not intended to assess recovery of injured organisms.

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 biased 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 a temperature appropriate for growth of the test microorganism(s) 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 Practice E645).

7.1.1 *Phosphate Buffer Solution, Stock*—Dissolve 34 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, must be used 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 At least three replicates of each test condition are required for these procedures. The number of additional replicates necessary to the evaluation is dictated by the statistical significance required for the expected results, 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	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.

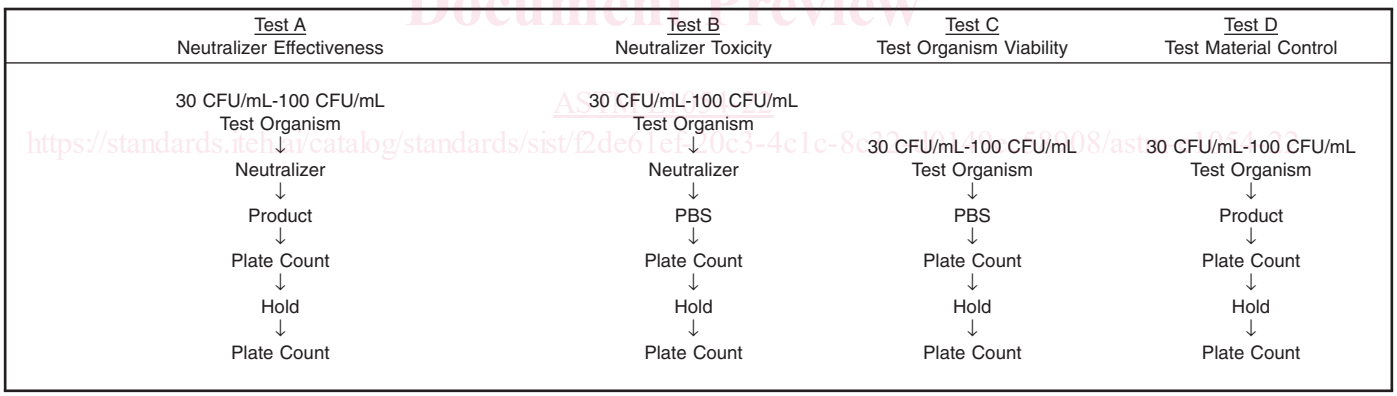

FIG. 1 Testing Schema for Neutralization Assay with Recovery on Semi-solid Medium

variability encountered in the data, and the relative effectiveness of the neutralization procedure.

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 CFU/mL 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 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 CFU/mL 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 CFU/mL 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 demonstrating 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 CFU/mL 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 CFU/mL to 100 CFU/mL of the microorganism (see Note 4).

Test A Neutralizer Effectiveness	Test B Neutralizer Toxicity	Test C Test Organism Viability	Test D Test Material Control
30 CFU/mL-100 CFU/mL Test Organism ↓ Neutralizer/Nutrient Medium ↓ Product ↓ Incubate ↓ Check for Growth	30 CFU/mL-100 CFU/mL Test Organism ↓ Neutralizer/Nutrient Medium ↓ PBS ↓ Incubate ↓ Check for Growth	30 CFU/mL-100 CFU/mL Test Organism ↓ Nutrient Medium ↓ PBS ↓ Incubate ↓ Check for Growth	30 CFU/mL-100 CFU/mL Test Organism ↓ Nutrient Medium ↓ Product ↓ Incubate ↓ Check for Growth

FIG. 2 Testing Schema for Neutralization Assay with Recovery in Liquid Medium

9.3.2 Add a volume of product, or solution containing product, to neutralizer-nutrient medium/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 representative of that used in the test.

9.3.3 Repeat this procedure (9.3.1 and 9.3.2) an additional four or more times, for a total of at least five replicates.

9.3.4 Incubate the product/neutralizer-nutrient medium/microbial suspension under the same conditions as those used in the antimicrobial effectiveness evaluation.

9.3.5 Following incubation, check for growth. If growth is present, record as a "1," and if no growth is present, as a zero. Confirm growth of the challenge microorganism by plating the suspension.

9.4 *Test B—Neutralizer Toxicity:*

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

9.4.2 Add a volume of PBS to the neutralizer-nutrient medium/microbial suspension in the same dilution ratio as that used for product in Test A (see 9.3.2).

9.4.3 Repeat this procedure (9.4.1 and 9.4.2) an additional four or more times, for a total of at least five replicates.

9.4.4 Incubate the PBS/neutralizer-nutrient medium/microbial suspension under the same conditions as those used in the antimicrobial effectiveness evaluation.

9.4.5 Following incubation, check for growth. If growth is present, record as a "1," and if no growth is present, as a zero. Confirm growth of the challenge microorganism by plating the suspension.

9.5 *Test C—Test Organism Viability:*

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

9.5.2 Add a volume of PBS or other appropriate buffering agent to the nutrient medium/microbial suspension in the same volume as that used for product in Test A (see 9.3.2).

9.5.3 Repeat this procedure (9.5.1 and 9.5.2) an additional four or more times, for a total of at least five replicates.

9.5.4 Incubate the PBS/nutrient medium/microbial suspension under the same conditions as those used in the antimicrobial effectiveness evaluation.

9.5.5 Following incubation, check for growth. If growth is present, record as a "1," and if no growth is present, as a zero. Confirm growth of the challenge microorganism by plating the suspension.

9.6 *Test D—Test Material Control (see Note 7):*

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

9.6.2 Add a volume of product to the nutrient medium/microbial suspension in the same volume as that used for product in Test A (see 9.3.2).

9.6.3 Repeat this procedure (9.6.1 and 9.6.2) an additional four or more times, for a total of at least five replicates.

9.6.4 Incubate the product/nutrient medium/microbial suspension under the same conditions as those used in the antimicrobial effectiveness evaluation.

9.6.5 Following incubation, check for growth. If growth is present, record as a "1," and if no growth is present, as a zero. Confirm growth of the challenge microorganism by plating the suspension.

10. Neutralization Assay with Recovery by Membrane Filtration (Fig. 3)

10.1 At least three replicates of each test condition are required for these procedures. The number of additional replicates necessary to the evaluation is dictated by the statistical significance required for the expected results, the variability encountered in the data, and the relative effectiveness of the neutralization procedure.

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

10.3 *Test A—Neutralization Effectiveness:*

10.3.1 Filter through the membrane a volume of product, or solution containing product, that is the same as that used in the antimicrobial effectiveness evaluation.

NOTE 8—This can be achieved by adding 1 mL of a microbial suspension containing 30 CFU/mL to 100 CFU/mL to the PBS.

NOTE 9—The membrane filter type and apparatus must be the same as

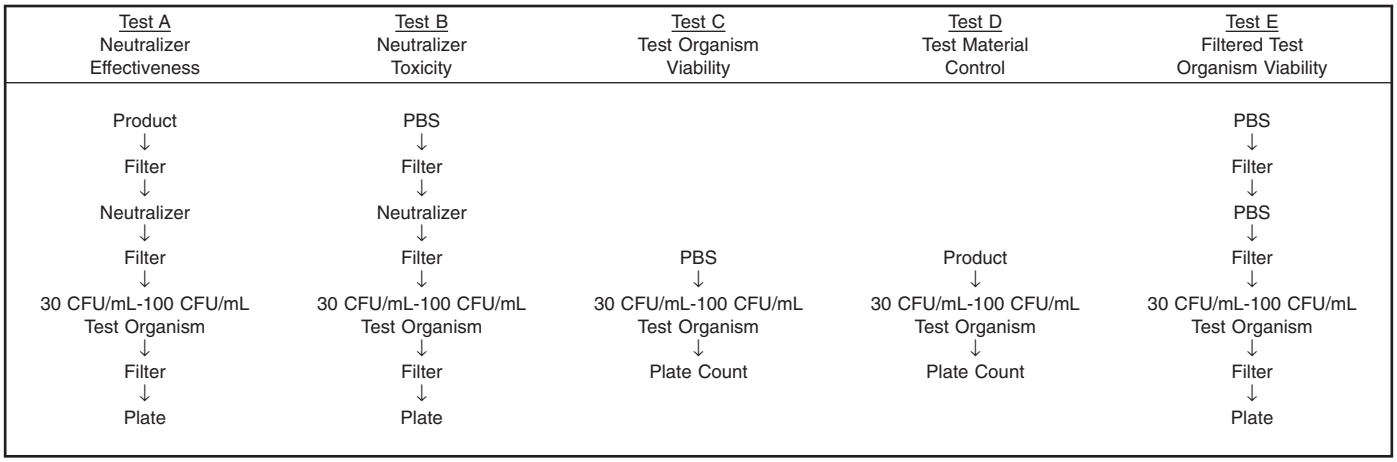

FIG. 3 Testing Schema for Neutralization Assay with Recovery by Membrane Filtration

that used in the antimicrobial effectiveness evaluation.

10.3.2 Filter through the membrane a volume of neutralizer that is the same as that used in the antimicrobial effectiveness evaluation.

10.3.3 Inoculate a volume of PBS, or other appropriate buffering agent, with a volume of the challenge microbial suspension such that the resulting suspension contains 30 CFU to 100 CFU of the microorganism (see Note 4). Filter the PBS/microbial suspension through the membrane.

10.3.4 Aseptically place the membrane filter on the surface of an appropriate nutrient agar medium or onto absorbent pads with appropriate nutrients. Use the same materials used in the antimicrobial effectiveness evaluation, including incorporated neutralizers, if appropriate.

10.3.5 Repeat this procedure (10.3.1 – 10.3.4) an additional two or more times, for a total of at least three replicates.

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

10.4 *Test B—Neutralizer Toxicity:*

10.4.1 Filter through the membrane a volume of PBS that is equal to the volume of product used in Test A (see 10.3.1).

10.4.2 Filter through the membrane a volume of neutralizer that is equal to the volume used in Test A (see 10.3.2).

10.4.3 Inoculate a volume of PBS, or other appropriate buffering agent, with a volume of the challenge microbial suspension such that the resulting suspension contains 30 to 100 CFU of the microorganism (see Note 4 and Note 8). Filter the PBS/microbial suspension through the membrane (see Note 9).

10.4.4 Aseptically place the membrane filter on the surface of an appropriate nutrient agar medium or onto absorbent pads with appropriate nutrients. Use the same materials used in Test A (see 10.3.4).

10.4.5 Repeat this procedure (10.4.1 – 10.4.4) an additional two or more times, for a total of at least three replicates.

10.4.6 Incubate the agar plates or pads under the same conditions as those in the antimicrobial effectiveness evaluation, and following incubation, enumerate the colony-forming units.

10.5 *Test C—Test Organism Viability:*

10.5.1 Inoculate a volume of PBS, or other appropriate buffering agent, with a volume of the challenge microbial suspension such that the resulting suspension contains 30 CFU/mL to 100 CFU/mL of the microorganism (see Note 4 and Note 8).

10.5.2 Within 1 min of execution of 10.5.1, 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.

10.5.3 Repeat this procedure (10.5.1 and 10.5.2) an additional two or more times, for a total of at least three replicates.

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

10.6 *Test D—Test Material Control (see Note 7):*

10.6.1 Inoculate the product with a volume of the challenge microbial suspension such that the resulting suspension contains 30 CFU/mL to 100 CFU/mL of the test organism (see Note 4 and Note 8).

10.6.2 Hold the product/microbial suspension for an exposure period necessary to allow detection of an antimicrobial effect (see Note 7).

10.6.3 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.

10.6.4 Repeat this procedure (10.6.1 – 10.6.3) an additional two or more times, for a total of at least three replicates.

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

10.7 *Test E—Filtered Test Organism Viability:*

10.7.1 Filter a volume of PBS through the membrane filter that is equal to the volume of product, or solution containing product, used in Test A (see 10.3.1).

10.7.2 Filter a volume of PBS through the membrane filter that is equal to the volume of neutralizer used in Test A (see 10.3.2).

10.7.3 Inoculate a volume of PBS, or other appropriate buffering agent, with a volume of the challenge microbial suspension such that the resulting suspension contains 30 CFU/mL to 100 CFU/mL of the microorganism (see Note 4 and Note 8). Filter the PBS/microbial suspension through the membrane (see Note 9).

10.7.4 Aseptically place the membrane filter onto the surface of an appropriate nutrient agar medium or onto absorbent pads with appropriate nutrients. Use the same materials used in Test A (see 10.3.4).

10.7.5 Repeat this procedure (10.7.1 – 10.7.4) an additional two or more times, for a total of at least three replicates.

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

11. Interpretation of Data

11.1 *Recovery on Semi-solid Medium:*

11.1.1 Apply the following formula to calculate the number of surviving challenge microorganisms resulting from each replicate of each test, namely, the immediate post-neutralized plates and the timed post-neutralized plates:

$$\text{number of survivors} = \frac{(\text{plate count 1} + \text{plate count 2})}{2}$$

11.1.2 Transform the number-of-survivor values to $\log_{10}$.

11.1.3 Statistically compare the number of survivors ($\log_{10}$) from Tests A, B, and D to the Test C organism viability population using a one-way ANOVA or Student's t-test with significance level $\alpha = 0.05$[3] and equivalency margin $\delta = 0.5$.

NOTE 10—Prior to performing the statistical analysis, the investigator must first establish the confidence level of the test. A 95 % confidence level (corresponding to a significance level $\alpha = 0.05$) is commonly used for biological data. The investigator must also determine the equivalence margin, δ, which is the mean difference between test results that is negligible and not of practical importance. An equivalency margin of 0.5 been previously used for microbiological assays.[4,5,6]

11.1.4 Neutralization is considered adequate at a 95 % confidence if the Test A recovery population is statistically equivalent to the Test C organism viability population (if the 90 % confidence interval for mean Test A – mean Test C is contained in [-0.5, 0.5]), and if the Test D recovery population is significantly smaller than the Test C organism viability population ($p \leq 0.05$).

NOTE 11—An equivalency test for arbitrary values of α and δ is performed at a 100 %×(1-α) confidence level by comparing a 100 % × (1-2α) confidence interval to [-δ, δ]).[7]

11.1.5 The neutralizing medium is considered non-toxic if the Test B recovery population is statistically equivalent to from the Test C organism viability population (if the 90 % confidence interval for mean Test B – mean Test C is contained in [-0.5, 0.5])

11.2 *Recovery in Liquid Medium:*

11.2.1 Transform the number of replicates that are positive for growth (P) out of the N replicates tested at a single dilution to estimate the mean number of surviving microorganisms per replicate using the Most Probable Number technique (MPN):

$$\text{number of survivors} = MPN = -\ln\left(\frac{N - P + 1/2}{N+1}\right) \quad (1)$$

11.2.2 Transform the number-of-survivor values to $\log_{10}$(MPN)

11.2.3 Calculate the standard error (SE) of the $\log_{10}$(MPN) from a single dilution:

$$SE = \log_{10}(e)\sqrt{\frac{(1 - e^{-MPN})^2}{P \times MPN^2 \times e^{-MPN}}} \quad (2)$$

11.2.4 Use $\log_{10}$(MPN) and SE to statistically compare the mean numbers of survivors from Tests A, B, and D to the number of survivors from Test C per procedures described in 11.1.3–11.1.5.

11.2.5 When there are 10, 15 or 20 replicates in each Test group A, B and C, 90 % confidence intervals for the difference in mean $\log_{10}$-transformed number of survivors were calculated for all possible combinations of positives in two Test groups to assess equivalence, see Appendix X2, Table X2.1, Table X2.2, and Table X2.3.

11.3 *Recovery by Membrane Filtration:*

11.3.1 Apply the following formula to calculate the number of surviving challenge microorganisms resulting from each replicate of Test C and Test E:

$$\text{number of survivors} = \frac{(\text{plate count 1} + \text{plate count 2})}{2}$$

11.3.2 Transform the number-of-survivor values to $\log_{10}$ values.

11.3.3 Compare statistically the equivalence of numbers of survivors from Tests A, B, and D to the Test C Viability Population per procedures described in 11.1.3–11.1.5

12. Keywords

12.1 antimicrobial agents; antimicrobial effectiveness evaluations; inactivation; neutralization; neutralizer toxicity

[3] Dixon, W. J., and Massey, F. J., *Introduction to Statistical Analysis*, McGraw-Hill Book Co., NY, 1983, p. 678.

[4] Parker, A., D. Walker, D. Goeres, N. Allan, M. Olson, and A. Omar. Ruggedness and reproducibility of the MBEC biofilm disinfectant efficacy test. *J Microbiol Methods*, 102: 2014, pp 55-64.

[5] Fritz, B., D. Walker, D. Goveia, A. Parker, and D. Goeres. Evaluation of Petrifilm Aerobic Count plates as an equivalent alternative to drop plating on R2A agar plates in a biofilm disinfectant efficacy test. *Current Microbiol*, 70(3): 2015, pp 450-456.

[6] Safety and Effectiveness of Health Care Antiseptics; Topical Antimicrobial Drug Products for Over-the-Counter Human Use, A Rule by the Food and Drug Administration, Fed. Reg. 82(243): 60487, 21 CFR 310, 20 Dec 17

[7] Wellek, S. *Testing Statistical Hypotheses of Equivalence and Noninferiority*, 2nd Ed. CRC Press, Boca Raton, FL. 2010. 432 p.