Designation: E2197 – 17

Standard Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals[1]

This standard is issued under the fixed designation E2197; 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.

INTRODUCTION

The quantitative test method described here uses disks of stainless steel (1 cm in diameter) as carriers. It employs the same basic set of materials and procedures to assess the ability of liquid chemicals to inactivate vegetative bacteria, viruses, fungi, mycobacteria, and bacterial spores (**1-7**).[2] Performance standards for test substances, the level of water hardness, the type and level of a soil load, the test organism(s), and other test conditions may vary depending on the target regulatory agency. This basic test can also be adapted for use with other carrier materials of similar dimensions.

The development of this test method was made possible with financial support from the Antimicrobials Division of the U.S. Environmental Protection Agency.

1. Scope

1.1 This test method is designed to evaluate the ability of test substances to inactivate vegetative bacteria, viruses, fungi, mycobacteria, and bacterial spores (**1-7**) on disk carriers of brushed stainless steel that represent hard, nonporous environmental surfaces and medical devices. It is also designed to have survivors that can be compared to the mean of no less than three control carriers to determine if the performance standard has been met. For proper statistical evaluation of the results, the number of viable organisms in the test inoculum should be sufficiently high to take into account both the performance standard and the experimental variations in the results.

1.2 The test protocol does not include any wiping or rubbing action. It is, therefore, not designed for testing wipes.

1.3 This test method should be performed by persons with training in microbiology in facilities designed and equipped for work with infectious agents at the appropriate biosafety level (**8**).

1.4 It is the responsibility of the investigator to determine whether Good Laboratory Practice Regulations (GLPs) are required and to follow them where appropriate (40 CFR, Part 160 for EPA submissions and 21 CFR, Part 58 for FDA submissions).

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

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

1.7 *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:*[3]

A967/A967M Specification for Chemical Passivation Treatments for Stainless Steel Parts

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

[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] The boldface numbers in parenthesis refer to the list of references at the end of this standard.

[3] 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.

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *CFR Standard:*[4]

21 CFR, Part 58 Laboratory Practice for Nonclinical Laboratory Studies

40 CFR, Part 160 Good Laboratory Practice Standards

2.3 *CEN Standard:*[5]

EN 10088-2 1J/2J Stainless steels - Part 2: Technical delivery conditions for sheet/plate and strip of corrosion resisting steels for general purposes

3. Terminology

3.1 *Definitions*—For definitions of general terms used in this test method, refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *carrier, n*—an inanimate surface or object inoculated with the test organism.

3.2.2 *eluate, n*—an eluent, which contains the recovered organism(s).

3.2.3 *eluent, n*—any solution that is harmless to the test organism(s) and that is added to a carrier to recover the organism(s) in or on it.

3.2.4 *neutralization, n*—a process to quench the antimicrobial activity of a test substance. This process may be achieved by dilution of the organism/test substance mixture and/or by adding to it one or more chemical neutralizers.

3.2.5 *soil load, n*—a solution of one or more organic, or inorganic substances, or both, added to the suspension of the test organism to simulate the presence of body secretions, excretions, or other extraneous substances.

3.2.6 *test organism, n*—an organism that has characteristics that allow it to be readily identified. It also may be referred to as a surrogate, a simulant, or a marker organism.

3.2.7 *test substance, n*—a formulation that incorporates antimicrobial ingredients.

4. Summary of Test Method

4.1 Each disk (1 cm in diameter) receives 10 µL of the test organism with a soil load. The inoculum is dried, and then the disk is placed on the inside bottom surface of a sterile plastic vial prior to contact with 50 µL of the use-dilution of test substance. The contact time and temperature may vary as required. Control carriers receive 50 µL of a fluid harmless to the test organism(s) and its host cells, if any, but are otherwise treated in the same way as test carriers.

4.2 For tests against vegetative bacteria, fungi, mycobacteria, and bacterial spores, the test substance is then neutralized and the inoculum eluted. The eluate and subsequent rinses of the carrier and its vial are membrane filtered. Culture plates with the filters are incubated, colonies counted, and $\log_{10}$ reductions calculated.

4.3 For tests with viruses, appropriate dilutions of the eluate are inoculated into suitable cell cultures, the cultures are examined for cytopathology/infectious foci, which are estimated as the most probable number (MPN) or counted as foci or plaques, and $\log_{10}$ are calculated.

5. Significance and Use

5.1 The design of this test eliminates any loss of viable organisms through wash off, thus making it possible to produce statistically valid data using many fewer test carriers than needed for methods based on simple MPN estimates.

5.2 The stringency in the test is provided by the use of a soil load, the microtopography of the brushed stainless steel carrier surface, and the smaller ratio of test substance to surface area typical for many disinfectant applications. Thus, the test substance being assessed is presented with a reasonable challenge while allowing for efficient recovery of the test organisms from the inoculated carriers. The metal disks in the basic test are also compatible with a wide variety of actives.

5.3 The design of the carriers makes it possible to place onto each a precisely measured volume of the test organism (10 µL) as well as the control fluid or test substance (50 µL).

5.4 The inoculum is placed at the center of each disk whereas the volumes of the test substance covers nearly the entire disk surface, thus virtually eliminating the risk of any organisms remaining unexposed.

5.5 In all tests, other than those against viruses, the addition of 10 mL of an eluent/diluent gives a 1:200 dilution of the test substance immediately at the end of the contact time. While this step in itself may be sufficient to arrest the microbicidal activity of most actives, the test protocol permits the addition of a specific neutralizer to the eluent/diluent, if required. Except for viruses, the membrane filtration step also allows processing of the entire eluate from the test carriers and, therefore, the capture and subsequent detection of even low numbers of viable organisms that may be present. Subsequent rinsing of the membrane filters with saline also reduces the risk of carrying any inhibitory residues over to the recovery medium. Validation of the process of neutralization of the test substance is required by challenge with low numbers of the test organism.

5.6 In tests against viruses, addition of 1 mL of buffer at the end of the contact time achieves a 1:20 dilution of the test substance while keeping the volume of the eluate reasonably small to allow for the titration of most or all of the eluate in cell cultures. Confirmation of neutralization of the test substance is required by challenge of a residual disinfection load with low numbers of infective units of the test virus. Since the virus assay system is indirect, an additional step is required to demonstrate that prior exposure of the appropriate cell line to any residual disinfectant or disinfectant/neutralizer mixture does not interfere with the detection of a low level of virus challenge (See Appendix).

NOTE 1—In 5.5 and 5.6, volumes of 10 mL and 1 mL are recommended instead of 9.95 mL and 950 µL, respectively, for ease of dispensing the eluent.

[4] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

[5] Available from European Committee for Standardization (CEN), Avenue Marnix 17, B-1000, Brussels, Belgium, http://www.cen.eu.

5.7 The soil load in this test is a mixture of three types of proteins (high molecular weight proteins, low molecular weight peptides, and mucous material) designed to represent body secretions, excretions, or other extraneous substances that microbicidal chemicals may encounter under field conditions. It is suitable for working with all types of test organisms included here. The components of the soil load are readily available and subject to much less variability than animal sera.

5.8 If distilled water or other diluent is not to be specified on the product label, the diluent for the test substance is assumed to be tap water. Since the quality of tap water varies considerably both geographically and temporally, this test method incorporates the use of water with a specified and documented level of hardness to prepare use-dilutions of test substance that require dilution in water before use. While water with a hardness of at least 300 ppm as $CaCO_3$ is recommended consult local regulations regarding use of hard water prior to testing.

5.9 The Annex contains a list of those organisms that are often used in assessing the microbicidal activities of disinfectants for use on environmental surfaces or medical devices. Culture conditions for each organism are also included in the Annex. Depending on the label claim(s) desired and the requirements of the target regulatory agency, one or more of the organisms listed may be selected for the testing. If organisms other than those listed are to be used (for example, in the dairy or brewing industries), a clear justification must be provided and details of the culture media and growth conditions must be validated and clearly specified in test reports.

6. General Equipment and Labware

6.1 *Air Displacement Pipettes,* Eppendorf or equivalent, 100 to 1000 µL with disposable tips.

6.2 *Analytical Balance,* to weigh chemicals and to standardize inoculum delivery volumes by pipettes.

6.3 *Cell Culture Flasks and Other Plastic-ware for Viruses,* (see Note 2) plastic cell culture flasks of 25- and 75-cm^2 capacity for culturing cells and for preparing virus pools; 12-well or 96-well plastic plates for titrating virus infectivity.

NOTE 2—Plastic culture ware may be purchased from most laboratory supply houses.

6.4 *Centrifuge,* to allow for the sedimentation of the cells/spores of the test organism(s) for concentration, or washing, or both.

6.5 *Colony Counter,* for example, Quebec Colony Counter.

6.6 *Desiccator,* recommended size is 25 cm wide by 20 cm deep, with an active desiccant for drying the inocula on the carriers.

6.7 *Dissecting Microscope,* for the screening of the metal disks for damage to surface topography.

6.8 *Environmental Chamber or Incubator,* to hold the carriers at the desired test temperature.

6.9 *Filter Sterilization System for Media and Reagents,* a membrane or cartridge filtration system (0.22-µm pore diameter) is required for sterilizing heat-sensitive solutions.

6.10 *Forceps,* straight or curved, (*1*) with smooth tips to handle membrane filters, and (*2*) to pick up the metal disk carriers for placement in plastic vials.

6.11 *Freezers,* a freezer at –20 ± 2°C is required for the storage of media and additives. A second freezer at –70°C or lower is required to store the stocks of test organisms.

6.12 *Glassware,* 1-L flasks with a side-arm and appropriate tubing to capture the filtrates from 47-mm diameter membrane filters; 250-mL Erlenmeyer flasks for culture media.

6.13 *Hemocytometer,* for counting fungal conidia, and/or for use in the preparation of suitable cell numbers for seeding monolayers.

6.14 *Hot Air Oven,* an oven at 60°C to dry clean and sterile glassware.

6.15 *Incubators,* an ordinary incubator, an anaerobic incubator, and a CO_2 incubator to incubate cell cultures in a 5 % CO_2 atmosphere. If only one ordinary incubator is available, its temperature will require adjustment depending on the type of organism under test.

6.16 *Inverted Microscope,* an inverted microscope with 10× eyepiece and 5×, 10×, and 40× objectives to examine cell cultures.

6.17 *Laminar Flow Cabinet,* a Class II (Type A) biological safety cabinet. The procedures for the proper maintenance and use of such cabinets are given in Ref (**8**).

6.18 *Liquid Nitrogen Storage for Cells,* a proper liquid nitrogen container and liquid nitrogen supply for cryopreservation of the stocks of cell lines.

6.19 *Magnetic Stir Plate and Stir Bars,* large enough for a 5-L beaker or Erlenmeyer flask for preparing culture media or other solutions.

6.20 *Markers,* for permanent marking of labware.

6.21 *Membrane Filtration System for Capture of the Test Organisms other than Viruses,* sterile 47-mm diameter membrane filters (0.22- or 0.45-µm pore diameter) and glass, plastic, or metal holders for such filters are required.

6.22 *pH Meter,* to measure pH of buffers, eluents, and test formulations.

6.23 *Microwave Oven,* to melt agar overlays.

6.24 *Miscellaneous Laboratory Ware,* pipette tips, plastic vials for storing cell and viral stocks, dilution tubes.

6.25 *Orbital Shaker,* for shaking the broth cultures of *Bacillus subtilis* during their incubation.

6.26 *Petri Plates (Pyrex glass) 150 mm in diameter,* for holding and autoclave sterilization of metal disks.

6.27 *Positive Displacement Pipette,* a pipette and pipette tips fitted with "plungers" that can accurately dispense 10-µL volumes for inoculation of carriers without the aerosol generation that occurs when air displacement pipettes are used.

6.28 *Refrigerator,* a refrigerator at 4 ± 2°C for storage of media, culture plates and reagents.

6.29 *Serological Pipettes,* sterile reusable or single-use pipettes of 10.0, 5.0, and 1.0 mL capacity.

6.30 *Spectrophotometer,* for measuring turbidity of microbial suspensions.

6.31 *Sterile Dispenser,* 10 mL, for dispensing diluent/eluent.

6.32 *Sterile Disposable Gloves,* for handling the carriers.

6.33 *Sterile Disposable Plastic Petri Dishes,* 100 by 15 mm.

6.34 *Sterile Polypropylene Centrifuge Tubes with Caps,* 50-mL.

6.35 *Sterilizer,* any steam sterilizer suitable for processing culture media, reagents, and labware is acceptable. The steam supplied to the sterilizer must be free from additives toxic to the test organisms or cell cultures.

6.36 *Timer,* any stopwatch that can be read in minutes and seconds.

6.37 *Vacuum Source,* a vacuum pump, access to an in-house vacuum line or a water faucet vacuum apparatus required to pull the samples through the membrane filters.

6.38 *Vials (Glass),* wide-mouth, 20-mL, for use as dilution vials.

6.39 *Vials (Nalgene),* wide-mouth, 30-mL, for holding the inoculated carriers to be exposed to the test formulation.[6]

6.40 *Vortex Mixer,* to vortex the eluate and rinsing fluid in the carrier vials to ensure efficient recovery of the test organism(s).

NOTE 3—The method described here uses conventional membrane filters. The system with hydrophobic grid membrane filters (HGMF) may also be used for this purpose (**9**).

NOTE 4—It is important to analyze the whole sample to detect and count any survivors and ensure confidence in the data generated. For this reason, membrane filtration is usually superior to pour-plating or spread-plating, which normally can process only a small fraction of the volumes of eluates in this method.

7. General Solutions and Reagents

7.1 *Purity of Reagents,* Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (**10**).

7.2 Other chemical grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. For information on the testing of reagents not listed by the American Chemical Society, see (**11**) and (**12**).

7.3 *Absolute Alcohol,* in a 100-mL plastic or glass beaker for flame-sterilization of metallic forceps used to handle membrane filters.

7.4 *Cell Culture Media and Supplements for Working with Viruses*—(see Note 5) Culture media and the types and ratios of supplements will vary depending on the cell line used. Eagle's minimal essential medium (EMEM) with 5 to 10 % fetal bovine serum is used for growing a wide variety of cells. Please refer to other sources for further details on working with cell cultures (**13**) and viruses (**14**) and for preparing virus pools to be used in virucidal tests (**15**).

NOTE 5—Material and reagents for cell culture and virology may be purchased from biological supply houses.

7.5 *Phosphate Buffered Stock Solution*—To prepare a stock solution of phosphate buffer, 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.

7.6 *Phosphate Buffered Saline (PBS),* to be used as a diluent and wash for all organisms except viruses; to prepare PBS, add 1.25 mL of the stock solution and 8.75 g of NaCl to a volumetric flask, fill with deionized water to the 1000 mL mark, and mix; adjust pH to 7.2 ± 0.2, if necessary. Sterilize by filtration or autoclaving.

7.7 *Trypsin (1:250) for Work with Rotaviruses,* to be added at a final concentration of 5 µg/mL to maintenance media when making rotavirus pools or assaying for their infectivity.

NOTE 6—Trypsin preparations can vary in strength depending on the supplier and the degree of purity, and the concentration specified here is only a guide. Preliminary testing may be required to determine the optimal concentration for the specific type of product being used.

7.8 *Test Substance,* prepared at its use-dilution and brought to the test temperature. The number of lots of the test substance to be evaluated, and whether one or more of them is aged or not to simulate the shelf-life to be claimed, will depend on the target regulatory agency.

7.9 *Growth, Recovery Media, and Media Supplements,* the required types of materials (see below) can be purchased from a variety of sources specializing in laboratory supplies.

7.10 $MnSO_4 \cdot H_2O$, added to diluted Columbia broth to promote *B. subtilis* sporulation.

7.11 *Test Substance Diluent,* for test substances requiring dilution to obtain a use-dilution, water with a standardized and specified level of hardness, as $CaCO_3$, shall be used as the diluent.

7.12 *Deionized Distilled Water (DDW),* or equivalent high-quality water, for making reagent solutions and media. (See Terminology D1129 and Specification D1193.)

7.13 *Plates of Recovery Media for Bacteria and Fungi,* media must be prepared and sterilized according to manufacturer's instructions and then aseptically dispensed into culture plates. Sterility and growth promotion checks of media batches should always be performed as the included negative and positive controls.

7.14 *Earle's Balanced Salt Solution (EBSS),* pH of 7.2 to 7.4. To be used as diluent and wash for virus titration.

7.15 *Tryptone, Bovine Serum Albumin (BSA), and Bovine Mucin,* the three ingredients for the soil load (Section 9) can be purchased from a variety of chemical suppliers. The same level of yeast extract may be used in place of Tryptone.

[6] The sole source of supply of the apparatus (Nalgene vials, Catalog #2118-0001) known to the committee at this time is Nalge Nunc International, 75 Panorama Creek Dr., Rochester, N.Y. 14625-2385. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

7.16 *Eluent, PBS with 0.1% (w/v) Tween-80.* The eluent may contain additional ingredients to neutralize the active(s) in the test substance.

8. Carriers

8.1 *Stainless Steel Disks (1 cm in diameter and approximately 0.7 mm thick)*—The disks are prepared from sheets of magnetized and brushed stainless steel (AISI type 430) similar to that used in the manufacture of countertops.[7] See Note 7. A ground unidirectional finish obtained with 150 grit abrasive (AISI) meeting the specifications of a No. 4 Finish (EN 10088-2 1J/2J) is present on both sides of the carrier with rounded edges on the top side. The carriers should be passivated according to Specification A967/A967M which requires soaking in a mild acid bath (7% citric acid) for at least 20 min to remove any impurities and accumulated debris from the disk surface. To remove punching burrs from the edges of the discs, tumble in a barrel together with ceramic chips and a cleanser.

NOTE 7—Initially 430 stainless steel was recommended and used in the development of the method. While 430 steel may still be useful with many chemistries, recent data has shown a potential incompatibility with certain formulations and diluents leading to corrosion of the 430 steel and interference with efficacy. Use of 304 steel will avoid this corrosion effect.

8.1.1 New disks should be soaked in a detergent solution for at least one hour to degrease them and they can then be washed and sterilized by autoclaving. They can either be used once and discarded or used repeatedly with proper cleaning and sterilization in between. Avoid extended soaking of the disks in water or aggressive chemicals to reduce risk of corrosion or rusting.

8.1.2 If disks are to be reused, check each disk for pitting, rust, other damage or accumulated debris before use by screening under a dissecting microscope at a magnification of at least 20×. Discard those with visible damage to surface topography.

8.2 *Preparation of the Carriers*—Place a sheet of filter paper on the inside bottom surface of a glass petri dish (150 mm in diameter) and lay out up to 20 clean disks on it. Autoclave (45 min at 121°C) to sterilize the disks.

9. Soil Load

9.1 The soil load to be incorporated in the suspension of the test organism will consist of a mixture of the following stock solutions in PBS (pH 7.2):

9.2 Add 0.5 g of Tryptone or yeast extract to 10 mL of PBS.

9.3 Add 0.5 g of BSA to 10 mL of PBS.

9.4 Add 0.04 g of bovine mucin to 10 mL of PBS.

9.5 Prepare the solutions separately and sterilize by passage through a 0.22 µm pore diameter membrane filter, aliquot, and store at either 4 ± 2°C or –20 ± 2°C.

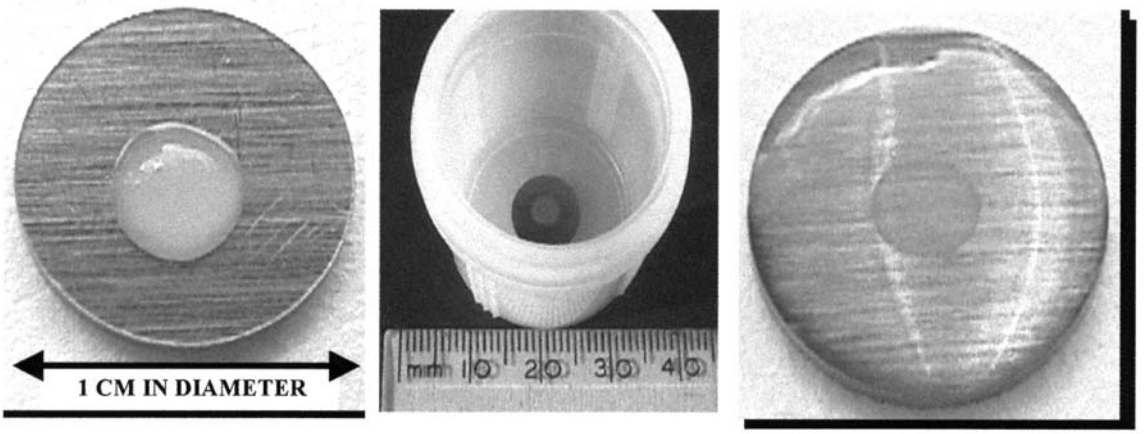
9.6 To obtain 500 µL of the inoculum, add 25 µL of BSA, 100 µL of mucin, and 35 µL of Tryptone or yeast extract stock to 340 µL of the microbial suspension.

NOTE 8—Animal sera, often used as a soil load, vary widely in their composition and may also contain microbial inhibitors. The soil load mixture given above contains a level of protein roughly equal to that in 5 % serum. Preliminary screening of albumin and mucin is recommended to ensure compatibility with test organism(s).

10. Preparation of Inocula

10.1 This test method can be used with most species of vegetative and spore-forming bacteria, viruses, fungi, and mycobacteria. The Appendix lists the organisms most often used. The number of CFU/mL of each freshly prepared and homogenized microbial test suspension, except viruses, may be estimated spectrophotometrically, based on a standard curve at

[7] The sole commercial source of supply of the stainless steel disks known to the committee at this time is Catalog P/N 304-107 from Pegen Industries, Inc. (#2-200 Iber Road, Ottawa, ON Canada K2SOL5; www.pegenindustries.com), but most competent machine shops could prepare such discs from the specifications. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.



NOTE 1—(A) Stainless disk inoculated with 10 µL of the test suspension, (B) The disk with the dried inoculum placed at the bottom of the vial, and (C) The disk with 50 µL of the test formulation over the dried inoculum.

FIG. 1 Inoculation and Handling of Stainless Steel Disks for Quantitative Carrier Test

a specific wavelength, but should be confirmed by titration using membrane filtration or alternative methods.

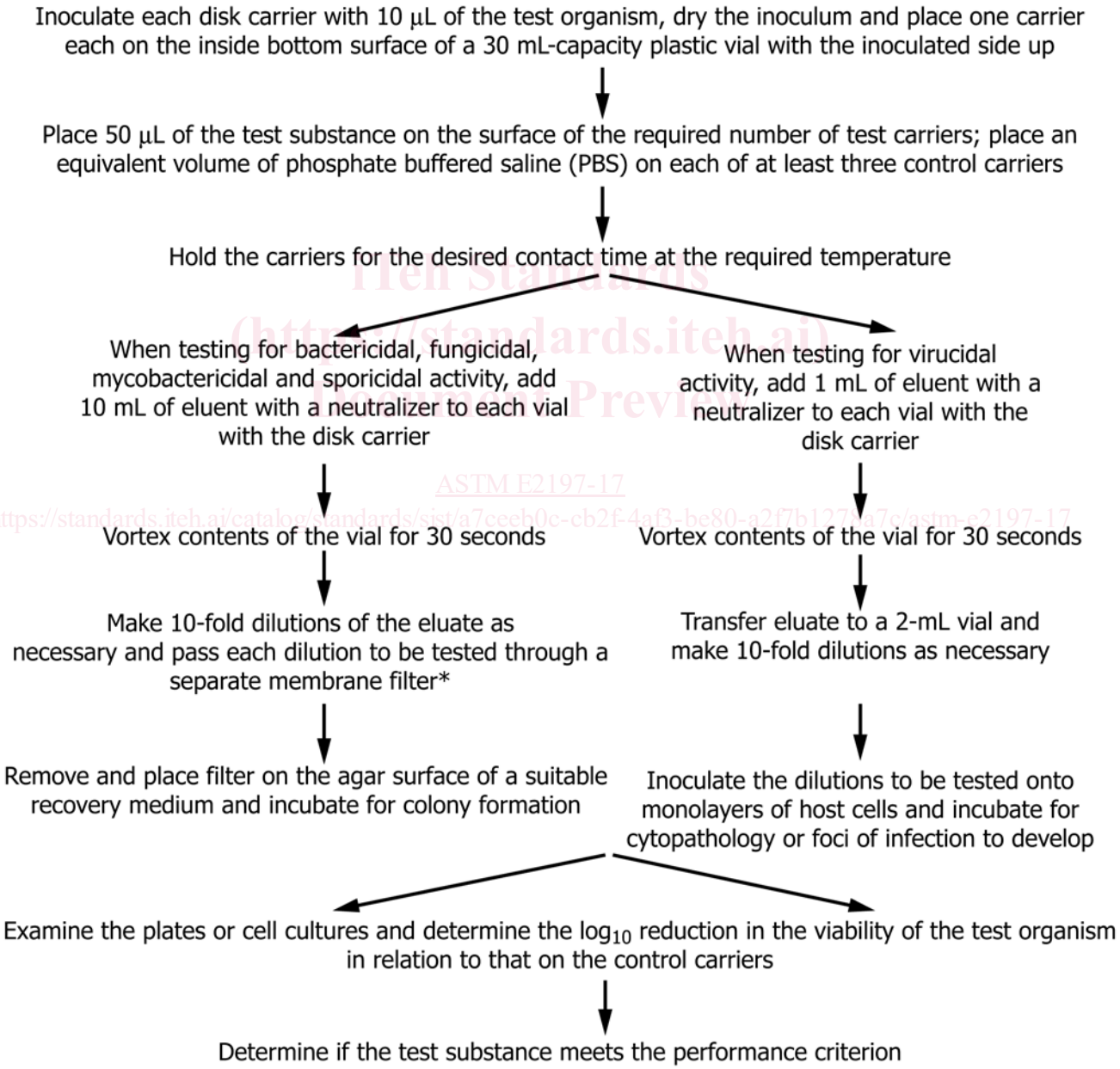
10.2 The concentration of the viable test organisms in the dried inoculum should be high enough to meet the required test substance performance criterion. In general, this number should not be more than 10× the defined performance standard. This should be confirmed in each test by determining the numbers of viable organisms on the control carriers.

11. Carrier Test (see Fig. 2)

11.1 Vortex the test suspension to evenly distribute cells, spores, or virus particles. Withdraw 10 µL of the suspension with a positive displacement pipette and place it at the center of each sterilized disk carrier without spreading the inoculum (Fig. 1A). (For consistency, the same pipette tip can be used throughout the inoculation of a batch of carriers). Cover the petri plate with its lid.

11.2 *Drying of the Inoculated Carriers*—Place the petri plate inside a desiccator and remove the lid of the petri plate. Cover the desiccator and make sure that it is properly sealed. Attach the outlet of the desiccator to a vacuum source and start evacuation of the air to achieve a vacuum of 20-25 in. mercury (508-635 torr; 677-847 mbar; 68000-85000 Pascal). Leave the disks in the evacuated desiccator at room temperature for two hours to dry.

NOTE 9—To avoid contamination of carriers with other organisms, it is



NOTE 1—*For spread-plating, the eluate is subjected to 10-fold dilutions as required and 0.1 mL of appropriate dilutions is separately spread on the surface of a suitable recovery medium, and incubated.

FIG. 2 Flow Chart Summary of Main Steps in Disk Carrier Test