Designation: E2197 – 11

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

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

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

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:*

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[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.

[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.

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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.

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 *Magnet,* strong enough to hold the disk carrier in place in the plastic vial while the liquid is being poured out of it for membrane filtration.

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

6.21 *Markers,* for permanent marking of labware.

6.22 *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.

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

6.24 *Microwave Oven,* to melt agar overlays.

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

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

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

6.28 *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.

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

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

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

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

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

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

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

6.36 *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.

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

6.38 *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.

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

6.40 *Vials (Nalgene),* wide-mouth, 30-mL, for holding the inoculated carriers to be exposed to the test formulation.[5]

6.41 *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.)

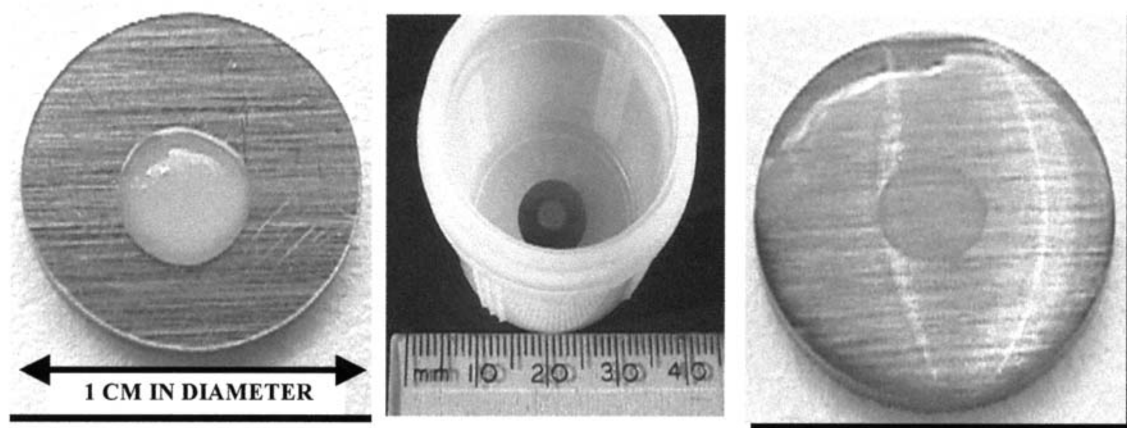
7.13 *Plates of Recovery Media for Bacteria and Fungi,* media must be prepared and sterilized according to manufacturer's instructions and then aseptically dispensed into culture plates. Sterility and growth promotion checks of media batches should always be performed as the included negative and positive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

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.

[5] 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.



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

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.[6]

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

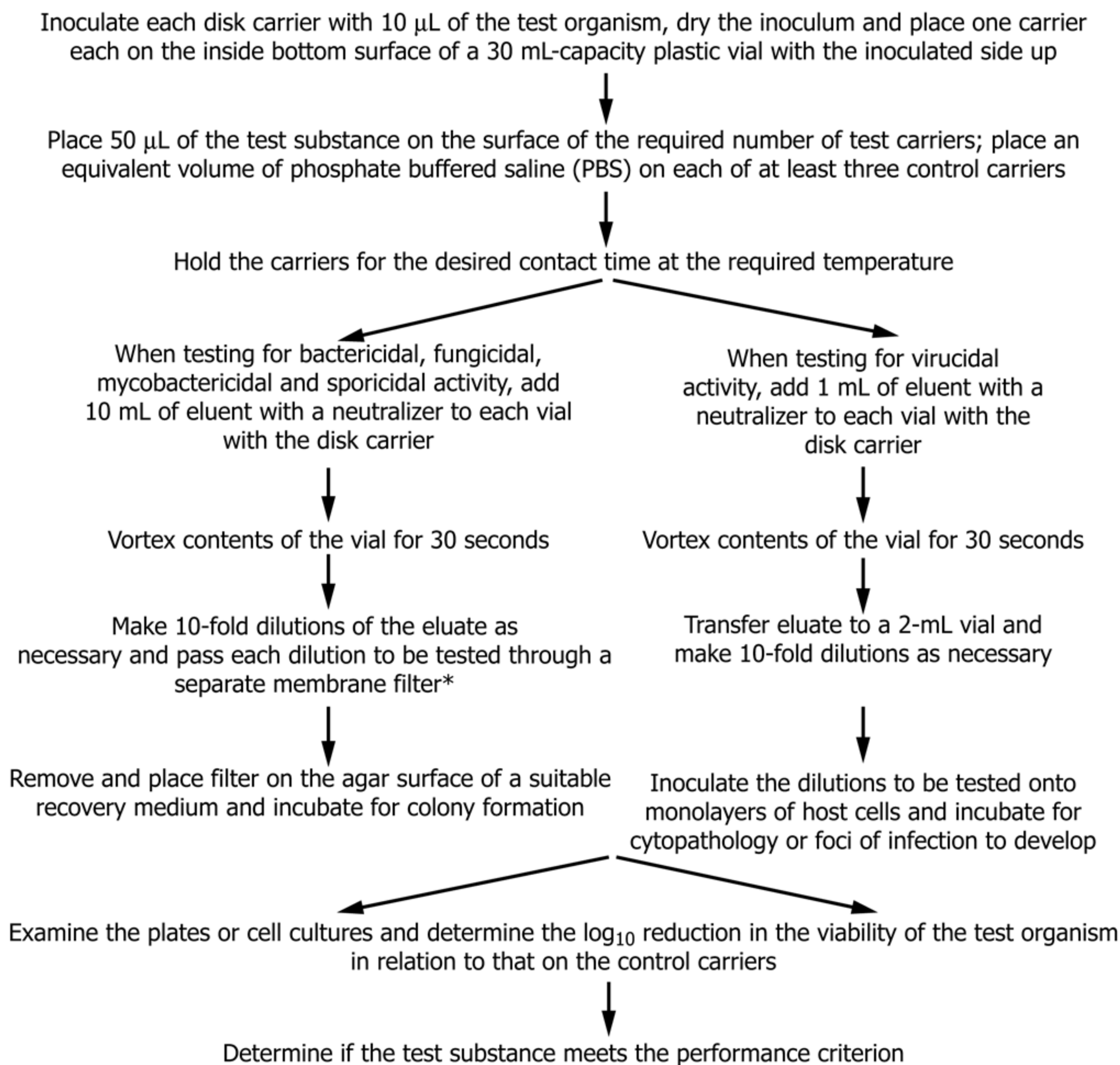
10.2 The concentration of the viable test organisms in the dried inoculum should be high enough to meet the required test substance performance criterion. In general, this number should not be more than 10× the defined performance standard. This should be confirmed in each test by determining the numbers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

[6] The sole commercial source of supply of the stainless steel disks known to the committee at this time is Muzeen & Blythe Ltd., 187 Sutherland Ave., Winnipeg, Manitoba, Canada R2W 3E6, 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—*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

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 8—To avoid contamination of carriers with other organisms, it is recommended to dedicate a desiccator for virus work only.

11.3 At the end of the drying period, remove the petri plate from the desiccator, cover the plate and observe the dried inoculum on each carrier. Discard any carrier in which the inoculum has run off the surface.

11.4 Carefully pick up each disk and place it, with the inoculated side up, on the inside bottom surface of a Nalgene vial (Fig. 1B). These carriers are now ready for the test procedure.

NOTE 9—Use at least three carriers as controls in each test and at least five test carriers for each lot of the test substance.

11.5 *Exposure of the Organism(s) to the Test Substance*—Cover the dried inoculum on each disk carrier with 50 µL of test substance (Fig. 1C) and hold the carriers at the desired temperature for the recommended contact period. Immediately at the end of the contact time, add 10 mL of the eluent alone or with a suitable neutralizer for all organisms except for viruses.

When testing for virucidal activity, add 1.0 mL of the eluent alone or with a suitable neutralizer to each vial containing the disk carrier.

11.6 Vortex the contents of the vial for 30 s.

11.7 Carefully examine each disk to ensure that the inoculum has been successfully eluted from it. In case of incomplete elution, continue vortexing as needed.

11.8 When working with organisms other than viruses, place a magnet on the outside bottom of the carrier vial to hold the disk in place, and pour the contents of the vial into the membrane filter holder. Rinse the carrier vial with about 15 mL of PBS, vortex, and filter the rinse. Repeat rinse three more times. Rinse the sides of the funnel unit with at least 40 mL of PBS. Aseptically transfer the membrane filter to the plate of a suitable recovery medium. Incubate the plates at the desired temperature for the required length of time (see Table 1).

11.9 When working with viruses, transfer the eluate to a 2-mL-capacity plastic vial and proceed with 10-fold dilutions using EBSS as the diluent. Inoculate the appropriate dilutions onto monolayers of suitable host cells and incubate at the desired temperature for the required period of time.

NOTE 10—Presence of high titers of viable organisms in eluates from the test carriers indicates no or weak activity of the test substance against the challenge organism(s) under the specific test conditions. To obtain a more precise indication of log_{10} reduction in viability by the test substance, assay of additional ten-fold dilutions of the eluates may be necessary (see Fig. 2).

NOTE 11—Separate filters, but the same filtration unit, can be used for a given carrier providing the dilutions are filtered in order starting with the most dilute.

12. Additional Controls in Virucidal Tests

12.1 The need for cell cultures when working with viruses requires the incorporation of additional controls because either the test substance or the neutralizer or a combination of both could alter the susceptibility of host cells to the virus under test. These controls must be run initially at least once and may not be included in subsequent tests as long as the same cell line, virus, test substance and neutralizer are being used for testing. The Appendix provides the details on these controls.

13. Calculating Log_{10} Reductions

13.1 A method for determining log_{10} reductions in the viability titer of the target organism by the test substance in quantitative carrier tests such as this one has been described(**16**).

14. Precision and Bias

14.1 *Precision*—The method has been subjected to extensive intralaboratory testing using a variety of test organisms to determine the extent of variability in the test data from operator to operator.

14.2 Target performance standards may vary depending on the target regulatory agency.

15. Keywords

15.1 adenovirus; *Aspergillus niger* conidia; *Bacillus subtilis* spores; bactericides; calicivirus; *Candida albicans*; cell cultures; chemical microbicides; *Clostridium sporogenes* spores; eluate; eluent; environmental surfaces; fungicides; hepatitis A virus; medical devices; membrane filtration; mycobactericides; *Mycobacterium terrae*; norovirus; parvovirus; *Pseudomonas aeruginosa*; quantitative carrier test; rhinovirus; rotavirus; soil load; sporicides; *Staphylococcus aureus*; standard hard water; surrogate; *Trichophyton mentagrophytes* conidia; virucides; viruses

ANNEX

(Mandatory Information)

A1. MICROORGANISMS COMMONLY USED IN TESTING ENVIRONMENTAL SURFACE AND MEDICAL DEVICE DISINFECTANTS, AND THEIR GROWTH AND RECOVERY MEDIA

A1.1 The organisms listed are among those commonly used to test the microbicidal activities of disinfectants to be used on environmental surfaces or medical devices. Which of these are to be selected to assess a given substance will depend on the label claim(s) to be made and also the requirements of the target regulatory agency. If an organism (for example, one relevant for the dairy or brewing industry), other than those given here is to be used, a clear justification must be given for its choice and full details on its culture and quantitation must also be included in any reports on such testing.

A1.1.1 ***Staphylococcus aureus:***

A1.1.1.1 *Materials:*

(1) Frozen stock of *S. aureus* (ATCC 6538).

(2) Growth medium—tubes of tryptic soy broth (TSB).

(3) Recovery medium—plates of tryptic soy agar (TSA).

(4) Sterile PBS.

NOTE A1.1—TSA and TPB, which are based on soybean-casein digests, were used in the development of the method described here. Other media with similar test formulations may be used instead but must be specified.

A1.1.1.2 *Method:*

(1) Prepare 100 mL of TSB according to the manufacturer's instructions and distribute aliquots of approximately 10 mL into the appropriate number of test tubes. Sterilize as per manufacturer's instructions.

(2) Inoculate a test tube of broth with 100 µL of thawed stock culture.

(3) Incubate for 18 ± 2 h at 35 ± 2°C (should yield $>10^9$ CFU/mL).

TABLE 1 Cultivation and Recovery of Various Test Organisms Recommended for Use in the Disk Carrier Test[A]

Organism (ATCC #)	Culture Medium or Cell Line	Recovery Medium or Cell Line
Staphylococcus aureus (6538)	Tryptic soy broth (TSB); incubation at 35 ± 2°C for 18 ± 2 h	Tryptic soy agar (TSA); plates read after 48 ± 2 h at 35 ± 2°C
Pseudomonas aeruginosa (15442)	TSB diluted 1:1000 with deionized distilled water; incubation at 35 ± 2°C for three days	TSA; plates read after 48 ± 2 h at 35 ± 2°C
Conidia of *Trichophyton mentagrophytes* (9533)	Sabouraud's Dextrose Agar (SDA); incubation for 12 days at 29 ± 2°C	SDA; plates observed first after 72 ± 2 h and final reading recorded after ten days at 29 ± 2°C
Candida albicans (10231)	SDB; incubation for 48 ± 4 h at 29 ± 2°C	SDA; plates observed first after 72 ± 2 h and final reading recorded after ten days at 29 ± 2°C
Conidia of *Aspergillus niger* (64958)	SDA; incubated for 12 days at 29 ± 2°C	SDA; plates observed first after 72 ±2 h and final reading recorded after five days at 29 ± 2°C
Mycobacterium terrae (15755)	Middlebrook 7H9 broth with glycerol and ADC enrichment; incubation at 35 ± 2°C for 21 days	Middlebrook 7H11 agar with OADC; plates observed after 14 days and weekly thereafter for a final reading after 30 days at 35 ± 2°C
Spores of *Bacillus subtilis* (19659)	Columbia broth diluted 1:10 with deionized distilled water; incubation for 72 h at 35 ± 2°C	TSA; plates observed daily and final reading recorded after five days at 35 ± 2°C
Spores of *Clostridium sporogenes* (7955)	Columbia broth; incubation at 29 ± 2°C under anaerobic conditions for five days	Fastidious anaerobic agar; plates observed first after 48 ± 2 h and final reading recorded after five days at 29 ± 2°C
Human Adenovirus 5 (VR-1516);	293 (CRL-1573)	Vero (CCL-81)
Hepatitis A Virus Strain HM-175 (VR-1402);	FRhK-4 (CRL-1688)	FRhK-4 (CRL-1688)
Canine Parvovirus– Strain Cornell 780916–80 (VR-2017);	A72 (CRL-1542)	A72 (CRL-1542)
Feline calicivirus F9 (VR-782)	CRFK (CCL-94)	CRFK (CCL-94)
Human Rhinovirus 37 (VR-1147) or 14 (VR-284)	MRC-5 (CCL-171) or WI-38 (CCL-75), or HeLa T^{4+}	MRC-5 (CCL-171) or WI-38 (CCL-75), or HeLa T^{4+}
Human Rotavirus strain Wa (VR-2018)	MA-104 (CRL-2378) or CV-1 (CCL-70)	MA-104 (CRL-2378) or CV-1 (CCL-70)
Murine Norovirus (strain S99 or MNV-1)	RAW 264.7 (TIB-71)	RAW 264.7 (TIB-71)

[A] For further details, please refer to the American Type Culture Collection's website at www.atcc.org.

(4) Standardize suspension as needed by diluting it with sterile PBS.

(5) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.2 *Pseudomonas aeruginosa:*

A1.1.2.1 *Materials:*

(1) Frozen stock of *P. aeruginosa* (ATCC 15442).

(2) Growth medium—tubes of TSB.

(3) Recovery medium—plates of TSA.

(4) Sterile PBS.

A1.1.2.2 *Method:*

(1) Prepare diluted TSB by adding 1 mL of regular TSB to 999 mL of DDW, distribute it in 10-mL aliquots in test tubes, and sterilize by autoclaving at 121°C for 20 min.

(2) Inoculate each tube of broth with 100 µL of thawed stock culture.

(3) Incubate for three days at 35 ± 2°C (should yield about 10^8 CFU/mL).

(4) Centrifuge suspension at 5000 × *g* for 15 min and resuspend pellet in 1⁄10 initial volume of TSB.

(5) Standardize suspension as needed by diluting it with sterile PBS.

(6) Prior to inoculation of carriers, the soil load is added as described in Section 9.

NOTE A1.2—This method has been validated using diluted TSB for growing *P. aeruginosa* and use of other liquid media may affect the stringency of the test protocol.

A1.1.3 *Trichophyton mentagrophytes:*

A1.1.3.1 *Materials:*

(1) Stock culture of *T. mentagrophytes* (ATCC 9533).

(2) Growth and recovery media—plates of Sabouraud's dextrose agar (SDA).

(3) Plates of Sabouraud's Dextrose Agar as growth and recovery media.

(4) Sterile stainless steel spatula.

(5) Sterile PBS.

(6) 250-mL flask with glass beads (sterile).

(7) Sterile absorbent cotton.

(8) Sterile 150-mL glass beaker.

(9) Hemocytometer to count fungal conidia.

A1.1.3.2 *Method:*

(1) Transfer a loopful (10 µL) of the stock culture of *T. mentagrophytes* to the center of each of four Sabouraud's dextrose agar plates.

(2) Incubate plates at 29 ± 2°C for 12 days (not less than ten days and not more than 15 days).

(3) Remove mycelial mats from the surface of the agar plates using a sterile spatula: four plates might provide a sufficiently high number of conidia.

(4) Transfer to 250-mL flask containing 25 to 50 mL PBS with glass beads; shake flask vigorously enough to break off the conidia from the hyphae.

(5) Filter suspension through sterile absorbent cotton into a beaker (conidia are collected in the filtrate in the beaker).

(6) Estimate density of conidial suspension by counting in hemocytometer.

(7) Standardize suspension as needed by diluting it with sterile PBS.

(8) Conidial suspensions stored at 2 to 10°C can be used for up to four weeks as test inocula in fungicidal tests.

(9) Maintain stock culture of fungus on a Sabouraud's dextrose agar plate at 4 ± 2°C. At three-month intervals, inoculate a fresh agar plate and incubate plate for ten days at 29 ± 2°C.

(10) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.4 *Candida albicans:*

A1.1.4.1 *Materials:*

(1) Frozen stock of *Candida albicans* (ATCC 10231).

(2) Growth medium—tubes of Sabouraud's dextrose broth (SDB).

(3) Recovery medium—plates of SDA.

(4) Sterile PBS.

A1.1.4.2 *Method:*

(1) Prepare 100 mL of Sabouraud's dextrose broth according to the manufacturer's instructions and distribute aliquots of approximately 10 mL into the appropriate number of test tubes. Sterilize as per manufacturer's instructions.

(2) Inoculate a test tube of broth with 100 µL of thawed stock culture.

(3) Incubate for 48 ± 4 h at 29 ± 2°C.

(4) Standardize suspension as needed by diluting it with sterile PBS.

(5) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.5 *Aspergillus niger:*

A1.1.5.1 *Materials:*

(1) Stock culture of *A. niger* (ATCC 64958).

(2) Growth and recovery media—plates of SDA.

(3) Sterile stainless steel spatula or swab.

(4) Sterile PBS containing 0.05% polysorbate 80.

(5) Sterile PBS.

(6) 250-mL flask with glass beads (sterile).

(7) Sterile absorbent cotton.

(8) Sterile 150-mL glass beaker.

(9) Hemocytometer to count fungal conidia.

A1.1.5.2 *Method:*

(1) Transfer a loopful of the stock culture of *A. niger* to the center of each of four SDA plates.

(2) Incubate plates at 29 ± 2°C for 10 days (not less than 7 days and not more than 12 days).

(3) Suspend conidia by adding 3- to 5-mL of PBS containing 0.05 % polysorbate-80 to the surface of the agar plates and detaching them using a sterile spatula or swab: four plates will provide a sufficiently high number of conidia.

(4) Transfer the suspension to a 250-mL flask containing 15 to 40 mL PBS with glass beads; shake flask vigorously enough to disperse spores.

(5) Filter suspension through sterile absorbent cotton into a beaker (conidia are collected in the filtrate in the beaker).

(6) Estimate density of conidial suspension by counting in hemocytometer.

(7) Standardize suspension as needed by diluting it with sterile PBS.

(8) Conidial suspensions stored at 2 to 10°C can be used for up to four weeks to inoculate carriers.

(9) Maintain stock culture of fungus on an SDA plate at 4 ± 2°C. At three-month intervals, inoculate a fresh agar plate and incubate plate for seven days at 29 ± 2°C.

(10) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.6 *Mycobacterium terrae:*

A1.1.6.1 *Materials:*

(1) Frozen stock of *M. terrae* (ATCC 15755).

(2) Growth medium—flasks of sterile Middlebrook 7H9 broth containing glycerol and ADC Enrichment.

(3) Recovery medium—plates of Middlebrook 7H11 Agar supplemented with OADC.

(4) Sterile PBS.

(5) Sterile bijoux bottles with ten glass beads (5 mm in diameter) in each.

(6) Plastic cell culture flasks (75 cm^2) with a canted neck and a cap with a 0.2 µm filter in it.

A1.1.6.2 *Method:*

(1) Place 100 mL of sterile 7H9 broth to each of two culture flasks.

(2) Inoculate each flask of broth with 1 mL of thawed stock culture.

(3) Incubate for 21 days at 35 ± 2°C in a stationary state.

(4) Centrifuge suspension at 5000 × *g* for 15 min and decant supernatant.

(5) Wash by resuspending in sterile PBS.

(6) Repeat centrifugation and washing steps a total of three times.

(7) Transfer the pellet to a bijoux bottle with glass beads and sterile PBS and vortex to break up clumps of the cells (the suspension should contain no less than 10^9 CFU/mL).

(8) Standardize suspension as needed by diluting it with sterile PBS.

(9) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.7 *Bacillus subtilis:*

A1.1.7.1 *Materials:*

(1) Frozen stock of *B. subtilis* (ATCC 19659).

(2) Growth medium—flasks of sterile Columbia Broth diluted 1:10 with sterile deionized water.

(3) Recovery medium—plates of TSA.

(4) Sterile 10 mm $MnSO_4$ 4 H_2O.

(5) Sterile PBS.

(6) Orbital platform shaker.

A1.1.7.2 *Method:*

(1) Place 99 mL of 1⁄10 Columbia broth and 1 mL of 10 mM $MnSO_4$ 4 H_2O solution in a sterile culture flask.

(2) Inoculate the flask of broth with 100 µL of thawed stock culture.

(3) Incubate for 72 ± 2 h at 35 ± 2°C on an orbital shaker and shake at 150 rpm (should produce approximately 10^{-8} viable spores/mL).

(4) Centrifuge suspension at 5000 × *g* for 20 min at 4°C and decant supernatant.

(5) Wash by resuspending in sterile PBS.

(6) Repeat centrifugation and washing steps a total of three times.

(7) After the last centrifugation, resuspend the pellet in sterile PBS using 1⁄10 the volume of the original culture medium.

(8) Standardize suspension as needed by diluting it with sterile PBS.

(9) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.8 *Clostridium sporogenes:*

A1.1.8.1 *Materials:*

(1) Frozen stock of *C. sporogenes* (ATCC 7955).

(2) Growth medium—flasks of sterile full-strength Columbia broth.

(3) Recovery medium—plates of fastidious anaerobic agar (FAA).

(4) Sterile PBS.

(5) Anaerobic incubator.

A1.1.8.2 *Method:*

(1) Place 100 mL of sterile full-strength Columbia broth in a sterile culture flask.

(2) Inoculate the flask of broth with 100 µL of thawed stock culture.

(3) Incubate under anaerobic conditions for five days at 29 ± 2°C (should produce approximately 10^8 viable spores/mL).

(4) Centrifuge suspension at 5000 × *g* for 20 min at 4°C and decant supernatant.

(5) Wash by resuspending in sterile PBS.

(6) Repeat centrifugation and washing steps a total of three times.

(7) After the last centrifugation, resuspend the pellet in sterile PBS using 1⁄10 the volume of the original culture medium.

(8) Standardize suspension as needed by diluting it with sterile PBS.

(9) Prior to inoculation of carriers, the soil load is added as described in Section 9.

A1.1.9 *Cell Cultures:*

A1.1.9.1 *Materials:*

(1) Cell culture media.

(2) Supplements for the culture media.

(3) Cell culture flasks and other glass- and plasticware.

(4) CO_2 incubator set at either 33 ± 2°C or 36 ± 2°.

A1.1.9.2 *Method:*

(1) Trypsinize confluent monolayers of cells to maintain stocks as well as for titration of virus infectivity.

A1.1.10 *Viruses:*

A1.1.10.1 The selection of the test viruses for this method is based on their (*1*) relative safety for the laboratory staff, (*2*) ability to grow to titers sufficiently high for testing, (*3*) ability to produce cytopathic effects or plaques, or both, in cell cultures, (*4*) potential to spread through contaminated environmental surfaces and medical devices, and (*5*) relative resistance to a variety of chemicals. Other strains or types of viruses may be substituted provided they meet the preceding criteria. Depending on the regulatory agency and the types of claims to be made, testing against two or more of the following viruses may be required.

NOTE A1.3—There is insufficient information on whether the passage history, culture conditions, and strain differences of viruses can influence their susceptibility or resistance to chemical microbicides. Caution must be exercised, however, when substituting viruses as this may lead to variations in results from one laboratory to another.

(1) **Human adenovirus 5—**(ATCC VR-1516)—Recommended cell lines for making virus pools and infectivity titrations are 293 (CRL-1573) and Vero (ATCC CCL-81), respectively.

(2) **Hepatitis A virus strain HM-175—**(ATCC VR-1402)—Recommended cell line is FRhK-4 (ATCC CRL-1688).

(3) **Canine parvovirus strain Cornell 780916–80—**(ATCC VR-2017)—Recommended cell line is A72 (ATCC CRL-1542).

(4) **Feline calicivirus Strain F9—**(ATCC VR-782)—Recommended cell line is CRFK (CCL-94).

(5) **Human rhinovirus 37—**(ATCC VR-1147) or **14** (ATCC VR-284)—Recommended cell lines are MRC-5 (ATCC CCL-171), WI-38 (ATCC CCL-75) or HeLa T^{4+}. (Incubation of infected cells at 33°C is required for optimal virus replication).

(6) **Human rotavirus strain Wa—**(ATCC VR-2018)—Recommended cell lines are MA-104 (CRL-2378) or CV-1 (ATCC CCL-70).

NOTE A1.4—Prior to rotavirus inoculation, cell monolayers must be washed at least twice with EBSS to remove the serum from the growth medium. All diluents, maintenance media, and agar overlays also must be free from serum. Most rotaviruses also require the presence of trypsin in the medium for growth and infectivity assays.

A1.1.11 *Murine norovirus (strain S99 or MNV-1):* Recommended cell line is RAW 264.7 (ATCC TIB-71).

A1.1.11.1 *Materials:*

(1) Frozen stock of the virus.

(2) Cultures of the appropriate cell line.

(3) EMEM with or without serum.

(4) EBSS.

A1.1.11.2 *Methods:*

(1) Remove growth medium from a 75-cm^2 flask of host cell monolayer, wash the monolayer with EBSS as necessary and inoculate with 100 µL of thawed virus suspension.

(2) Allow the inoculum to remain in contact with the cells for 60 to 90 min.

(3) Add maintenance medium (EMEM with or without serum) and incubate the flasks at the appropriate temperature until about 75 % of the monolayer shows virus-induced cytopathology.

(4) Freeze (–20°C) and thaw (room temperature) the contents of the flask at least three times to release virus from infected cells.

(5) Centrifuge the contents of the flask at 1000 × *g* for 10 min and collect the supernatant. The supernatant may require ultra-centrifugation to concentrate the virus.

(6) Prior to inoculation of carriers, the soil load is added as described in Section 9.

APPENDIX

(Nonmandatory Information)

X1. ADDITIONAL CONTROLS REQUIRED WHEN ASSESSING THE VIRUCIDAL ACTIVITY OF A TEST SUBSTANCE

X1.1 Cytotoxicity Control

X1.1.1 *Objective*—The objective of this control is to determine the dilution of the test substance at which it causes no apparent degeneration (cytotoxicity) of the cell line to be used for measuring virus infectivity and assess if the neutralizer in any way reduces or enhances such cytoxicity.

X1.1.2 *Procedure:*

X1.1.2.1 Make an initial 1:20 dilution and one further ten-fold dilution of the use-dilution of the test substance in the eluent with and without the neutralizer.

X1.1.2.2 Remove the culture medium from the monolayers of the host cell line(s) and put into each test monolayer separately the same volume of inoculum (for example, 100 µL) used in the virus titration; control monolayers receive an equivalent amount of EBSS (without any neutralizer) only.

X1.1.2.3 Hold the cultures for 30 to 60 min at 36 ± 1°C and examine them under an inverted microscope for any apparent cytotoxicity.

X1.1.2.4 In case of cytotoxicity, a different neutralizer or alternative approaches to the removal/reduction of cytotoxicity, such as gel filtration, may be needed.

X1.1.2.5 If no cytotoxicity is observed at either one of the dilutions, the test substance and the neutralizer should be subjected to the interference test (see below).

X1.2 Validation of Neutralization of Microbicides Used in Tests for Virucidal Activity

X1.2.1 *Objective*—To determine if the neutralization of a test substance at the end of the contact time is sufficient to render it inactive against the test virus.

NOTE X1.1—The details given here are based mainly on the use of plaque assays to measure virus infectivity and may not lend themselves as readily to other methods for virus titration. However, validation of neutralization/quenching of virucidal activity and removal/reduction of cytotoxicity are integral to testing of microbicides against viruses. Those laboratories that do not use plaque assays must, therefore, provide detailed documentation on how they have addressed these issues for proper interpretation of the results.

X1.2.2 *Procedure:*

X1.2.2.1 Dilute test virus to give approximately 500 to 1000 infectious units/mL.

X1.2.2.2 Prepare the test substance at the level it is to be tested (use-dilution) for virucidal activity.

X1.2.2.3 Prepare a further 1:20 and a 1:200 dilution of the use-dilution in the neutralizer to be validated.

X1.2.2.4 Add 100 µL of the diluted virus separately to 900 µL of the 1:20 and 1:200 dilutions of the use-dilution as prepared in X1.2.2.3.

X1.2.2.5 As controls, add the same volume of the virus separately to 900 µL of the neutralizer alone and EBSS.

X1.2.2.6 Allow the vials to stand at room temperature for 5 min.

X1.2.2.7 Inoculate each host cell monolayer separately with the test and control solutions as prepared in X1.2.2.4 and X1.2.2.5.

X1.2.2.8 After virus adsorption, place a liquid or semi-solid maintenance overlay in the inoculated cultures and incubate them to allow for the development of plaques or cytopathology.

X1.2.3 *Possible Outcomes:*

X1.2.3.1 *Lack of Sufficient Neutralization of Virucidal Activity*—When compared to controls, a recovery of less than 80 ± 5 % of virus infectivity lower level of virus infectivity in the cultures inoculated with the mixture of test substance/neutralizer would indicate insufficient neutralization. This would require additional testing to improve the neutralization process.

X1.2.3.2 *Successful Neutralization of Virucidal Activity*—The presence of comparable levels of infectivity in cultures inoculated with the test substance/neutralizer mixture and the controls would mean successful quenching of virucidal activity and thus a validated neutralization process.

X1.2.3.3 *Cytotoxicity*—Apparent degeneration of the cell monolayers receiving the test substance/neutralizer mixture and the neutralizer alone would indicate cytotoxicity. The

presence of such cytotoxicity precludes the proper detection of any infectious virus. This will require the use of gel filtration to remove the cytotoxicity and re-titration of the samples for virus infectivity.

X1.3 Test to Check for Interference of Test Substance with Virus Infectivity

X1.3.1 *Objective:*

X1.3.1.1 To determine if sub-cytotoxic levels of the test substance can reduce or enhance virus infectivity in host cells.

X1.3.1.2 Levels of the test substance which show no obvious cytotoxicity could still reduce or enhance the ability of the challenge virus to infect or replicate in host cells, thus interfering with the estimation of its virucidal activity. An interference control must, therefore, be included to rule out such a possibility.

X1.3.1.3 While other means of assessing virus infectivity can be used in this test, plaque assays are considered the most appropriate. Therefore, the following protocol is being described with plaque assays as the recommended procedure.

X1.3.2 *Procedure:*

X1.3.2.1 Dilute test virus to give approximately 500 to 1000 infectious units/mL.

X1.3.2.2 Prepare the test substance at the level it is to be tested for virucidal activity.

X1.3.2.3 Prepare a further 1:20 and a 1:200 dilution of the test substance in the neutralizer to be validated.

X1.3.2.4 Put 100 µL of the 1:20 and 1:200 dilutions of the test product in the neutralizer into three wells each of a 12-well cell culture plate.

X1.3.2.5 In the remaining six wells, place 100 µL of the neutralizer in three and 100 µL of EBSS in three as controls.

X1.3.2.6 Incubate plate for 30 min.

X1.3.2.7 Observe monolayers seeded with host cells under an inverted microscope for any obvious signs of toxicity. If damage to the cells is readily visible, it is a sign of cytotoxicity. Use gel filtration to remove cytotoxicity.

X1.3.2.8 If monolayers show no observable damage, proceed with the next step to assay for interference with plaque formation.

X1.3.2.9 Wash the monolayers once with EBSS and add virus (diluted to give countable plaques in each well) to 9 wells which had separately received the product (6) and the neutralizer (3). To two of the remaining wells add virus with countable number of plaques. Leave the last well as cell culture control.

X1.3.2.10 Incubate plates for 60 to 90 min to allow virus to adsorb.

X1.3.2.11 Add overlay to the monolayers.

X1.3.2.12 Incubate plates at the appropriate temperature for the development of the virus plaques.

X1.3.3 *Possible Outcomes:*

X1.3.3.1 *Interference with Virus Infectivity*—Any interference by residual amounts of the test substance will result in significantly lower numbers of plaques in monolayers pretreated with sub-cytotoxic dilutions of the test substance when compared to the number of plaques in the control monolayers. Those dilutions that are toxic to the cells or do not exhibit virus replication, or both are not included in the $\log_{10}$ reduction calculations of the virucidal activity.

X1.3.3.2 *Enhancement of Virus Infectivity*—In this case, a higher than 45 ± 5 % in the number of plaques in the monolayers treated with a sub-cytotoxic level of the test substance (as compared to the controls) would indicate either disaggregation of virus clumps due to surfactants in the mixture or the alteration of virus receptors on the host cells.

X1.3.3.3 *Lack of Interference*—An absence of any interference with virus infectivity would be indicated by the appearance of similar numbers of plaques in treated and control monolayers.

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