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# Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides[1]

This standard is issued under the fixed designation E 2197; 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. Because it employs the same basic set of materials and procedures to assess the ability of liquid chemical germicides to inactivate vegetative bacteria, viruses, fungi, mycobacteria and bacterial spores **(1,2)**[2] it unifies the test methodology against a wide array of microorganisms. Performance standards for the categories of products to be tested, and the specific types of organism(s) to be used may vary depending on the regulatory agency. This basic test can also be adapted for use with other carrier materials of similar dimensions.

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

## 1. Scope

1.1 The method is designed to evaluate the ability of liquid chemical germicides to inactivate vegetative bacteria, viruses, fungi, mycobacteria and bacterial spores in the presence of a soil load **(1,2)** on disk carriers that represent environmental surfaces and medical devices. It is also designed to have survivors that can be compared to 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 size of the test inoculum should be sufficiently large to take into account both the performance standard and the experimental variation in the results.

1.2 The test protocol does not include any wiping or rubbing action. It is, therefore, not designed for testing germicide-soaked 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 **(3)**.

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

1.5 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).

1.6 *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:*

D 1129 Terminology Relating to Water[3]

D 1193 Specification for Reagent Grade Water[3]

E 1054 Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfectant, Sanitizer, Antiseptic, or Preserved Products[3]

E 2111 Standard Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal and Sporicidal Potencies of Liquid Chemical Germicides[4]

2.2 *CFR Standard:*

40 CFR, Part 160; 21 CFR, Part 58[5]

2.3 *Other Documents:*

Disinfectants (Chapter 6), Official Methods of Analyses (1998)[6]

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides 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] *Annual Book of ASTM Standards*, Vol 11.01. and 11.05

[4] *Annual Book of ASTM Standards*, Vol 11.05

[5] Available from Superintendent of Documents, U.S. Government Printing Office, Washington D.C. 20402.

[6] Available from AOAC International, Washington, DC.

CAN/CGSB-2.161-97, Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices[7]

## 3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

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

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

3.1.3 *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.

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

3.1.5 *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.

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

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

## 4. Summary of Test Method

4.1 Each disk (1 cm in diameter) receives 10 µL of the test organism with a soil load, dried, and is then placed on the inside bottom surface of a sterile, 15 to 20-mL-capacity vial prior to contact with 50 µL of the use-dilution of test substance (germicide). The contact time and temperature may vary as required. Control carriers receive 50 µL of a fluid harmless to the test organism(s).

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

4.3 For tests with viruses, appropriate dilutions of the eluate are inoculated into suitable cell cultures, the cultures examined for cytopathology/infectious foci and $\log_{10}$ calculated.

## 5. Significance and Use

5.1 The design of this test minimizes 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 most probable number (MPN) estimates.

5.2 The stringency in the test is provided by the use of a soil load, the microtopography of the carrier surface and the small ratio of disinfectant to surface area typical for many disinfectant applications. Thus the formulation under test is presented with a reasonable challenge while allowing for efficient recovery of the test organisms from the inoculated carriers with or without their exposure to the test formulation. The metal disks used 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 precisely measured volume of the test organism (10 µL) as well as the test formulation (50 µL).

5.4 The inoculum is placed at the centrer of each disk whereas the volume of the test formulation covers nearly the entire disk surface thus eliminating the risk of any organisms remaining unexposed to the test formulation.

5.5 The relatively small ratio of 1:5 between the volume of the inoculum and that of the test formulation closely reflects many field applications of liquid chemical germicides.

5.6 In all tests other than those against viruses, the addition of 9.95 mL of an eluent/diluent gives a 1:200 dilution of the test formulation immediately at the end of the contact time. While this step in itself may be sufficient to arrest the germicidal activity of most formulations, the test protocol permits the addition of a specific neutralizer to the eluent/diluent, if required; 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 normal saline also reduces the risk of carrying any inhibitory residues over to the recovery medium. Confirmation of neutralization of the test formulation is required by challenge with low numbers of the test organism.

5.7 In tests against viruses, addition of 950 µL of Earle's balanced salt solution (EBSS) at the end of the contact time achieves a 1:20 dilution of the test formulation 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 formulation 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.

5.8 The soil load for use 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 the body secretions, excretions or other extraneous substances that chemical germicides 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.

5.9 If distilled water or other diluent is not to be specified on the product label, the diluent 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 products that require dilution in water before use. The U.S. Environmental Protection Agency's Scientific Advisory Panel (SAP) on Germicide Test Methodology has recommended the use of water with a standard

[7] Available from the Canadian General Standards Board, Ottawa, Ontario, Canada.

hardness of 400 ppm as $CaCO_3$.

5.10 Depending on the label claim desired and the requirements of the target regulatory agency, additional test organisms may be used. In such cases, the details of the culture media and 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*[8], 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.

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 in the preparation of suitable cell numbers for seeding cell monolayers.

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

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

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 for this work. The procedures for the proper maintenance and use of such cabinets are given in Ref **(3)**.

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 glass 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*, permanent labware marking pens.

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.

NOTE 1—The method described here uses conventional membrane filters. The system with hydrophobic grid membrane filters (HGMF) may also be used for this purpose **(4)**.

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

6.25 *Orbital Shaker*, for shaking the broth cultures of *B. 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 dispense accurately 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 (Teflon)*, wide-mouth, 15 mL, for holding the inoculated carriers to be exposed to the test formulation.[9]

NOTE 2—Glass vials, unless they are shatterproof, may break during the vortexing of the disk carriers and the use of such vials should be avoided when working with metal disks.

[8] Plastic culture ware may be purchased from most laboratory supply houses.

[9] The Teflon vials (40 mm high by 29 mm outside diameter) available from Cole-Parmer, Vernon Hills, IL.

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

## 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 **(5)**.

7.2 Other chemical grades may be used **(6)**, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

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*[10], 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 **(7)** and viruses **(8)** and for preparing virus pools to be used in virucidal tests **(9)**.

7.5 *Sterile Normal Saline (0.85 % NaCl)*, to be used as a diluent and wash for all organisms except viruses. The pH of the saline should be 7.2 to 7.4.

7.6 *Sterile Normal Saline (0.85 % NaCl) with 0.1 % (v/v) Tween 80 (Saline-T)*, to be used as an eluent for all organisms except viruses. The pH of saline-T should be 7.2 to 7.4.

7.7 *Trypsin (1:250) for Work with Rotaviruses*[9], to be added at a final concentration of 5 µg/mL to maintenance media when making rotavirus pools or assaying for their infectivity.

NOTE 3—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 Germicide*, prepared at its use-dilution and brought to the test temperature. The number of lots of the formulation to be tested, 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 Product Diluent*, for test products 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)*, for making reagent solutions and media.

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.

7.14 *Diluent for Virus Titration*, Earle's balanced salt solution (EBSS) with a pH of 7.2 to 7.4.

7.15 *Phosphate Buffer*, prepared according to the formulation given in Ref **(10)**. Adjust buffer pH to 7.2.

7.16 *Tryptone, Bovine Serum Albumin (BSA) and Bovine Mucin*, the three ingredients for the soil load (Section 9) can a purchased from a variety of chemical suppliers.

## 8. Carriers

8.1 *Stainless Steel Disks (1 cm in Diameter and Approx. 0.7 mm Thick)*—The disks are prepared from sheets of magnetized and brushed stainless steel[11] similar to that used in the manufacture of countertops.[12]

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 to reduce risk of corrosion or rusting.

8.1.2 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 20X. 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 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 phosphate buffer (pH 7.2):

9.2 Add 0.5 g of tryptone to 10 mL of phosphate buffer.

9.3 Add 0.5 g of BSA to 10 mL of phosphate buffer.

9.4 Add 0.04 g of bovine mucin to 10 mL of phosphate buffer.

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 to 340 µL of the microbial suspension, 25 µL of BSA, 100 µL of mucin, and 35 µL of tryptone stock.

NOTE 4—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

[10] Material and reagents for cell culture and virology may be purchased from biological supply houses.

[11] The sheets (A.I.S.I stainless type 430) can be purchased from Drummond McCall, Winnipeg, Manitoba.

[12] The sole source of supply of the stainless steel disks known to the committee at this time is Engineering Department, Rehabilitation Centre, Ottawa, ON, Canada. 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, which you may attend.

mycobacteria. Table 1 lists the species and strains of the test 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 membrane filtration.

10.2 The concentration of the viable test organism in the dried inoculum should be high enough to meet the required product performance criterion (Table 2). In general, this number should not be less than 10× the defined performance standard. This should be confirmed in each test by determining the numbers of viable organisms on the control carriers.

10.3 *Staphylococcus aureus*

10.3.1 *Materials*:

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

10.3.1.2 Tryptose phosphate broth (TPB).

10.3.1.3 Trypticase soy agar (TSA).

NOTE 5—TSA and TPB, which are based on soybean-casein digests, were used in the development of the method described here. Other media with similar formulations may be used instead.

10.3.2 *Method*:

10.3.2.1 Prepare 100 mL of TPB 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.

10.3.2.2 Inoculate a test tube of broth with 100 µL of thawed stock culture.

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

10.3.2.4 Refer to Section 9 for the soil load.

10.4 *Pseudomonas aeruginosa*

10.4.1 *Materials*:

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

10.4.1.2 TPB.

**TABLE 2 Drying of the Test Organisms on the Inoculated Carriers[A]**

| Test Organism | Drying of the Inoculum (23 ± 2°C)) |
|---|---|
| *S. aureus* | 2 h in a desiccator under vacuum |
| *P. aeruginosa* | 2 h in a desiccator under vacuum |
| Conidia of *T. mentagrophytes* | 2 h in a desiccator under vacuum |
| *M. terrae* | 2 h in a desiccator under vacuum |
| Spores of *B. subtilis* | 2 h in a desiccator under vacuum |
| Spores of *C. sporogenes* | 2 h in a desiccator under vacuum |
| Adenoviruses | 30 to 40 min in a laminar flow hood |
| Hepatitis A Virus | 30 to 40 min in a laminar flow hood |
| Canine Parvovirus | 30 to 40 min in a laminar flow hood |
| Rhinoviruses | 30 to 40 min in a laminar flow hood |
| Rotavirus | 30 to 40 min in a laminar flow hood |

[A] The ability of vegetative bacteria and viruses to survive during drying can vary depending on indoor air temperature and relative humidity. Therefore, drying should occur under ambient temperature; the drying times indicated here are as a general guide only and care must be taken to ensure that the inoculum on each carrier becomes visibly dry and yet has enough viable organisms on it to allow for a valid test. The desiccator must have an active drying agent in it and vacuum is to applied during the drying process.

10.4.1.3 TSA.

10.4.2 *Method*:

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

NOTE 6—This method has been validated using diluted TPB for growing *P. aeruginosa* and use of other liquid media may affect the stringency of the test protocol.

10.4.2.2 Inoculate each tube of broth with 100 µL of thawed stock culture.

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

10.4.2.4 Centrifuge suspension at 5000 xg for 15 min and resuspend pellet in 1/10 initial volume of TPB.

10.4.2.5 Refer to Section 9 for the soil load.

10.5 *Trichophyton mentagrophytes*

**TABLE 1 The Cultivation and Recovery of the Various Test Organisms to be used in the Carrier Test[A]**

| Organism (ATCC #) | Culture Medium or Cell Line | Recovery Medium or Cell Line |
|---|---|---|
| *Staphylococcus aureus* (6538) | Tryptose phosphate broth; incubation at 35 ± 2°C for 18 h | Trypticase soy agar; plates read after 48 h at 35 ± 2°C |
| *Pseudomonas aeruginosa* (15442) | Tryptose phosphate broth diluted 1:1000 with deionized distilled water; incubation at 35 ± 2°C for three days | Trypticase soy agar; plates read after 48 h at 35 ± 2°C |
| Conidia of *Trichophyton mentagrophytes* (9533) | Sabouraud's Dextrose Agar; incubation for 12 days at 29 ± 2°C | Sabouraud's Dextrose Agar; plates observed first after 72 h and final reading recorded after ten 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 weekly and 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 | Nutrient agar; 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 h and final reading recorded after five days at 29 ± 2°C |
| Human Adenovirus Type 4 (VR-4); | 293 cells (CRL-1573) | Vero (CCL-81) |
| Hepatitis A Virus Strain HM-175 (VR-1402); | FRhK-4 (CRL-1688) | FRhK-4 (ATCC CRL-1688) |
| Canine Parvovirus–Cornell Strain (VR-2017); | A72 (CRL-1542) | A72 (CRL-1542) |
| Human Rhinovirus Type 37 (VR-1147) or Type 14 (VR-284) | MRC-5 (CCL-171) or WI-38 (CCL-75), HeLa $T^{4+}$ | MRC-5 (CCL-171) or WI-38 (CCL-75), 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) |

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

10.5.1 *Materials*:

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

10.5.1.2 Plates of Sabouraud's Dextrose Agar as growth and recovery media.

10.5.1.3 Sterile stainless steel spatula.

10.5.1.4 Sterile normal saline.

10.5.1.5 250-mL flask with glass beads (sterile).

10.5.1.6 Sterile absorbent cotton.

10.5.1.7 Sterile 150-mL glass beaker.

10.5.1.8 Bunsen burner.

10.5.1.9 Incubator set at 29 ± 2°C.

10.5.1.10 Hemocytometer to count fungal conidia.

10.5.2 *Method*:

10.5.2.1 Streak a loopful (10 µL) of thawed stock culture of *T. mentagrophytes* at the center of each of four Sabouraud's Dextrose Agar plates.

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

10.5.2.3 Remove mycelial mats from the surface of agar plates using a sterile spatula.

10.5.2.4 Transfer to 250-mL flask containing 25 to 50 mL sterile saline-T with glass beads; shake flask vigorously enough to break off the conidia from the hyphae.

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

10.5.2.6 Estimate density of conidial suspension by counting in hemocytometer.

10.5.2.7 Standardize suspension as needed by diluting it with sterile saline so that it contains about $1 \times 10^7$ conidia/mL for germicidal testing.

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

10.5.2.9 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.

10.5.2.10 Refer to Section 9 for the soil load.

10.6 *Mycobacterium terrae*

10.6.1 *Materials*:

10.6.1.1 *M. terrae* (ATCC 15755) frozen stock.

10.6.1.2 Sterile deionized distilled water.

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

10.6.1.4 Sterile Middlebrook 7H9 broth containing glycerol and ADC Enrichment.

10.6.1.5 Middlebrook 7H11 Agar supplemented with OADC.

10.6.1.6 Plastic Cell Culture Flasks (75 $cm^2$) with a canted neck and a cap with a 0.2 µm filter in it.

10.6.1.7 Incubator set at 35 ± 2°C.

10.6.1.8 Black, gridded membrane filters 47 mm in diameter (0.45 µm pore diameter).

10.6.2 *Method*:

10.6.2.1 Place 100 mL of sterile 7H9 broth to each of four culture flasks.

10.6.2.2 Add 1 mL of thawed stock culture to each flask.

10.6.2.3 Incubate at 35 ± 2°C for 21 days.

10.6.2.4 Put 21-day-old culture of *M. terrae* grown in 7H9 broth in sterile centrifuge tubes.

10.6.2.5 Centrifuge at 5000 xg for 15 min.

10.6.2.6 Decant supernatant.

10.6.2.7 Wash by resuspending in sterile distilled water.

10.6.2.8 Repeat centrifugation and washing steps a total of three times.

10.6.2.9 Place the pellet in a bijoux bottle with ten glass beads (5-mm in diameter) and sterile distilled water and vortex it to break up clumps of the cells (the suspension should contain no less than $10^9$ CFU/mL.

10.6.2.10 Refer to Section 9 for the soil load.

10.7 *Bacillus subtilis*

10.7.1 *Materials*:

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

10.7.1.2 Sterile Columbia Broth diluted 1:10 with sterile deionized water.

10.7.1.3 TSA.

10.7.1.4 Sterile 10 mm $MnSO_4 \cdot 4\ H_2O$.

10.7.1.5 Incubator set at 35 ± 2°C.

10.7.1.6 Orbital platform shaker.

10.7.2 *Method*::

10.7.2.1 To 99 mL of 1/10 Columbia broth add 1 mL of 10 mM $MnSO_4\ 4 \cdot H_2O$ solution.

10.7.2.2 Add 100 µL of thawed bacterial culture to each 100 mL of the broth.

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

10.7.2.4 Wash spore suspension three times by centrifuging it at 5000 xg for 20 min at 4°C and resuspending the pellet in sterile deionized distilled water (DDW). After the last centrifugation, resuspend the pellet in DDW using 1/10 the volume of the original culture medium.

10.7.2.5 Refer to Section 9 for the soil load.

10.8 *Clostridium sporogenes*

10.8.1 *Materials*:

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

10.8.1.2 Sterile full-strength Columbia broth.

10.8.1.3 Fastidious anaerobic agar (FAA).

10.8.1.4 Anaerobic incubator set at 29 ± 2°C.

10.8.1.5 *Method*:

10.8.1.6 Add 100 µL of thawed bacterial culture to each 100 mL of the broth.

10.8.1.7 Incubate at 29 ± 2°C for five days (should produce approximately $10^8$ viable spores/mL).

10.8.1.8 Wash spore suspension three times by centrifuging it at 5000 xg for 20 min at 4°C and resuspending the pellet in sterile deionized distilled water (DDW). After the last centrifugation, resuspend the pellet in DDW using 1/10 the volume of the original culture medium.

10.8.1.9 Refer to Section 9 for the soil load.

10.9 *Cell Cultures*

10.9.1 *Materials*:

10.9.1.1 Cell culture media.

10.9.1.2 Supplements for the culture media.

10.9.1.3 Cell culture flasks and other glass- and plasticware.

10.9.1.4 $CO_2$ incubator set at either 33 ± 2°C or 36 ± 2°.

10.9.2 *Methods*:

10.9.2.1 Trypsinize confluent monolayers of cell cultures to maintain stocks as well as to put up cells for titration of virus infectivity.

10.10 *Viruses*—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*) property 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 chemical germicides. 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 7—There is insufficient information on whether the passage history, culture conditions, and strain differences of viruses can influence their susceptibility or resistance to chemical germicides. Caution must be exercised, however, when substituting viruses as this may lead to variations in results from one laboratory to another.

10.10.1 *Human Adenovirus Type 4 (ATCC VR-4)*—Recommended lines for making virus pools and infectivity titrations are 293 (CRL-1573) and Vero (ATCC CCL-81) cells, respectively.

10.10.2 *Hepatitis A Virus Strain HM-175 (ATCC VR-1402)*—Recommended cell line FRhK-4 (ATCC CRL-1688).

10.10.3 *Canine Parvovirus Cornell Strain (ATCC VR-2017)*—Recommended cell line A72 (ATCC CRL-1542).

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

10.10.5 *Human Rotavirus Wa (ATCC VR-2018)*—Recommended cell lines are MA-104 (CRL-2378) and CV-1 (ATCC CCL-70).

NOTE 8—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.

10.11 *Materials*:

10.11.1 Frozen stock of the virus.

10.11.2 Cultures of the appropriate cell line.

10.11.3 EBSS.

10.11.4 Refer to Section 9 for the soil load.

10.12 *Methods*:

10.12.1 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.

10.12.2 Allow the inoculum to remain in contact with the cells for 60 to 90 min.

10.12.3 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.

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

10.12.5 Centrifuge the contents of the flask at 1000 xg for 10 min and collect the supernatant. The supernatant may require ultra-centrifugation to concentrate the virus in it.

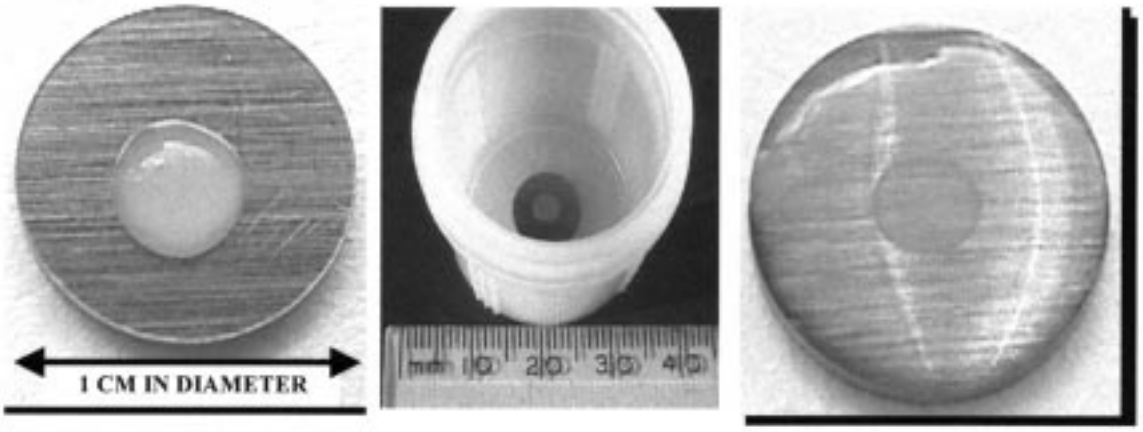
## 11. Carrier Test (see Fig. 2)

11.1 *Inoculation of the Carriers*—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 a disk carrier (Fig. 1A). For consistency, the same pipette tip can be used throughout the inoculation of a batch of carriers.

11.1.1 Allow the inoculum to become visibly dry. The length of the drying period and the actual method of drying will vary depending on the nature of the test organism (see Table 2).

11.1.2 Observe the dried inoculum on each carrier and discard any carrier in which the inoculum has run off the surface of the disk.

11.1.3 Carefully pick up each disk and place it, with the inoculated side up, on the inside bottom surface of a vial (Fig.




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

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

**Flow Chart**
**The Main Steps in the Disk Carrier Test**

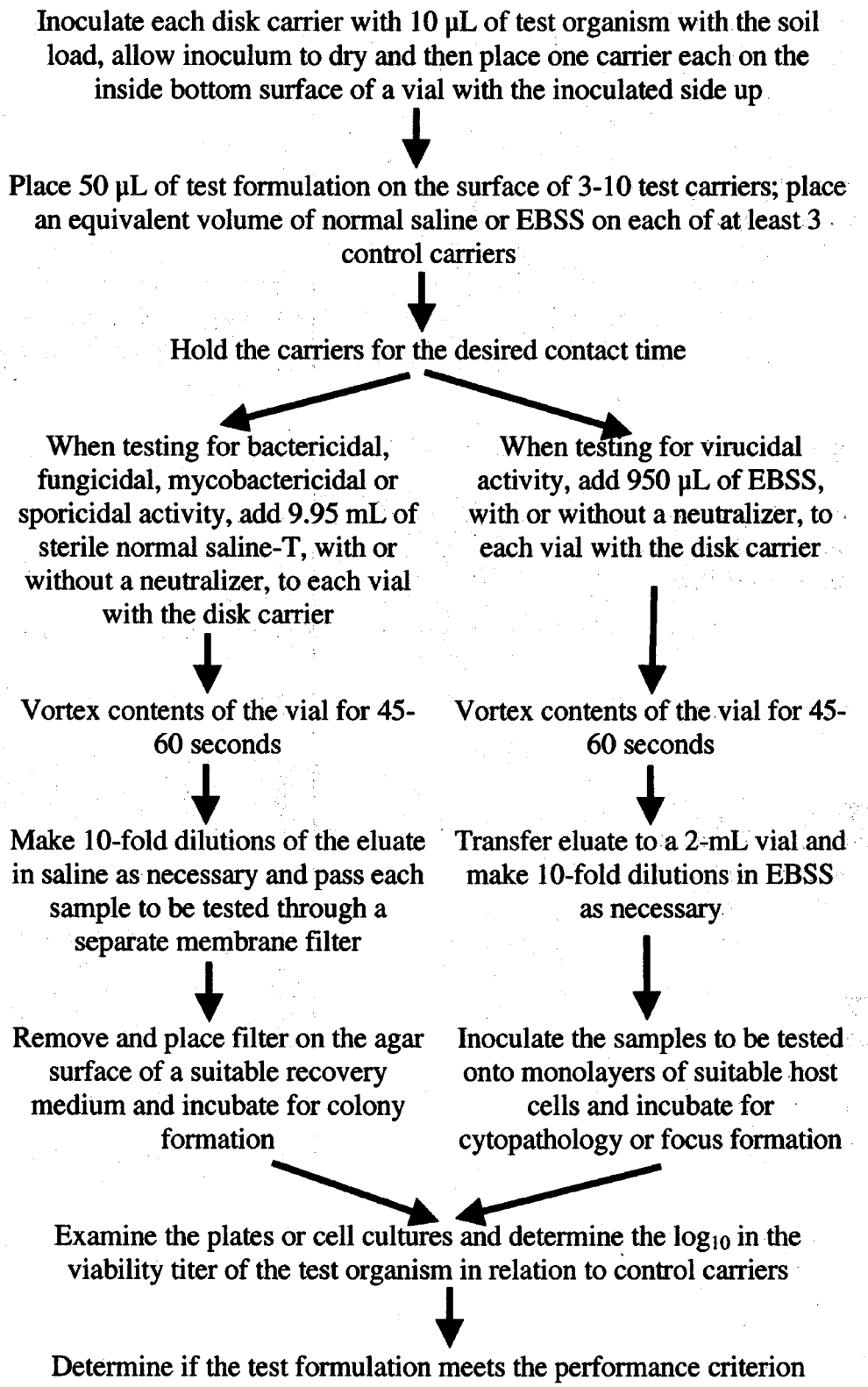



FIG. 2 Flow Chart—The Main Steps in the Disk Carrier Test

1B). These carriers are now ready for the test procedure.

NOTE 9—The number of test carriers to be used in each run is 10; however, in preliminary tests during product development only 3 to 5 test carriers may be sufficient to assess the potency of experimental formulation(s) against the test organism(s).

11.2 *Exposure of the Organism(s) to the Formulation Under Test*—Cover the dried inoculum on each disk carrier with 50 µL of test formulation (Fig. 1C) and hold the carriers at the desired temperature for the recommended contact period. Immediately at the end of the contact time, add 9.95 mL of sterile normal saline-T, with or without a neutralizer, for all organisms except for viruses. When testing for virucidal activity, add 950 µL of EBSS, with or without neutralizer, to each vial containing the disk carrier.

11.2.1 Vortex the contents of the vial for 45 to 60 s.

11.2.2 Carefully examine each disk to ensure that the inoculum has been successfully eluted from it. In case of any visible residue, scrape the inoculated side of the disk with the disposable tip of a pipette while flushing the disk surface several times with the eluent.

11.2.3 When working with organisms other than viruses, use a magnet 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 normal saline, vortex, and filter the rinse. Repeat rinse three more times. Rinse the sides of the funnel unit with at least 40 mL of saline. 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 2). In tests with viruses, place the eluate in 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 formulation against the challenge organism(s) under the specific conditions used of the test. To obtain a more precise indication of $\log_{10}$ reduction in viability by the test formulation, assay of additional ten-fold dilutions of the eluates may be necessary (see Fig. 2).

11.3 *Additional Controls in Virucidal Tests*—The need for cell cultures when working with viruses requires the incorporation of the following additional controls in tests for virucidal activity 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 formulation and neutralizer are being used for testing.

11.3.1 *Cytotoxicity Control*—The objective of this control is to (*1*) 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 (*2*) assess if the neutralizer in any way reduces or enhances such cytoxicity. Make an initial 1:20 dilution and one further ten-fold dilution of the use-dilution of the test substance in EBSS with and without the neutralizer, if any. 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 as used in virus titration; control monolayers receive an equivalent amount of EBSS (without any neutralizer) only. Hold the cultures for 30 to 60 min at room temperature and examine them under an inverted microscope for any cytotoxicity. In case of cytotoxicity, a different neutralizer or alternative approaches to the removal/reduction of cytotoxicity may be needed. If no cytotoxicity is observed at either one of the dilutions, the test substance and the neutralizer should be subjected to the following interference test.

11.3.2 *Control for Interference with Virus Infectivity*—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. Remove the culture medium from the host cells and inoculate each one of the test monolayers with the same volume of inoculum as used in virus titration with a 1:20 dilution of the test substance in EBSS with and without neutralizer. Controls receive EBSS alone (without the neutralizer). Hold the monolayers at room temperature for 30 to 60 min and inoculate each with a low number (20 to 50) of infective units of the challenge virus. Incubate the monolayers for virus adsorption, place maintenance medium in the cultures, incubate them for the time required for virus replication and examine them for cytopathology or foci of virus infection. Any significant difference in virus infectivity titer is indicative of the test substance's or the neutralizer's ability to affect the virus susceptibility of the host cells. In such a case, a different neutralizer or alternative approaches to the removal of the residues of the test product in the samples to be titrated for virus infectivity may be needed.

11.4 *Control Carriers*—The minimum number of control carriers to be used in each test is three regardless of the number of test carriers.

11.4.1 Instead of the test formulation, add 50 µL of sterile normal saline or EBSS to each control carrier. The contact time and temperature for the control carriers must be the same as that for the test carriers.

11.4.2 Follow the steps described in 11.2.2 to 11.2.5 as appropriate.

NOTE 11—Separate membrane filters, but the same filtration unit, can be used for a given carrier starting with the most dilute sample first.

## 12. Calculating $\text{Log}_{10}$ Reductions

12.1 A method for determining $\log_{10}$ in the viability titer of the target organism by the test formulation in quantitative carrier tests such as this one has been described **(11)**.

## 13. Precision and Bias

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

13.2 Target performance standards may vary depending on the regulatory agency.

## 14. Keywords

14.1 Adenovirus; *Bacillus subtilis* spores; bactericides; cell

cultures; chemical germicides; *Clostridium sporogenes* spores; eluate; eluent; environmental surfaces; fungicides; hepatitis A virus; medical devices; membrane filtration; mycobactericides; *Mycobacterium terrae*; *Pseudomonas aeruginosa*; quantitative carrier test; rhinovirus; rotavirus; soil load; sporicides; *Staphylococcus aureus*; standard hard water; surrogate; *Trichophyton mentagrophytes* conidia; virucides; viruses

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