



Designation: E2197 – 02

Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporocidal Activities of Liquid Chemical Germicides¹

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. 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:*³

D1129 [Terminology Relating to Water](#)

D1193 [Specification for Reagent Water](#)

E1054 [Test Methods for Evaluation of Inactivators of Antimicrobial Agents](#)

E2111 [Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal, and Sporocidal Potencies of Liquid Chemical Microbicides](#)

2.2 *CFR Standard:*

40 CFR, Part 160; 21 CFR, Part 58⁴

2.3 *Other Documents:*

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 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 Superintendent of Documents, U.S. Government Printing Office, Washington D.C. 20402.

Disinfectants (Chapter 6), Official Methods of Analyses (1998)⁵

CAN/CGSB-2.161-97, Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices⁶

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 center 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

⁵ Available from AOAC International, Washington, DC.

⁶ Available from the Canadian General Standards Board, Ottawa, Ontario, Canada.

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 hardness of 400 ppm as CaCO₃.

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*⁷, plastic cell culture flasks of 25 and 75-cm² 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 CO₂ incubator to incubate cell culture in a 5 % CO₂ 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.

⁷ Plastic culture ware may be purchased from most laboratory supply houses.

6.39 *Vials (Teflon)*, wide-mouth, 15 mL, for holding the inoculated carriers to be exposed to the test formulation.⁸

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.

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*⁹, 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*⁸, 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 *MnSO₄·H₂O*, 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 CaCO₃, 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 be 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 steel¹⁰ similar to that used in the manufacture of countertops.¹¹

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.

¹⁰ The sheets (A.I.S.I stainless type 430) can be purchased from Drummond McCall, Winnipeg, Manitoba.

¹¹ 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.

⁸ The Teflon vials (40 mm high by 29 mm outside diameter) available from Cole-Parmer, Vernon Hills, IL.

⁹ Material and reagents for cell culture and virology may be purchased from biological supply houses.

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 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.

TABLE 2 Drying of the Test Organisms on the Inoculated Carriers^A

Test Organism	Drying of the Inoculum (23 ± 2°C)
<i>S. aureus</i>	2 h in a desiccator under vacuum
<i>P. aeruginosa</i>	2 h in a desiccator under vacuum
Conidia of <i>T. mentagrophytes</i>	2 h in a desiccator under vacuum
<i>M. terrae</i>	2 h in a desiccator under vacuum
Spores of <i>B. subtilis</i>	2 h in a desiccator under vacuum
Spores of <i>C. sporogenes</i>	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.

10.3.2.2 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 >10⁹ 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.

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

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
<i>Staphylococcus aureus</i> (6538)	Tryptose phosphate broth; incubation at 35 ± 2°C for 18 h	Trypticase soy agar; plates read after 48 h at 35 ± 2°C
<i>Pseudomonas aeruginosa</i> (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 <i>Trichophyton mentagrophytes</i> (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
<i>Mycobacterium terrae</i> (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 <i>Bacillus subtilis</i> (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 <i>Clostridium sporogenes</i> (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 ⁺	MRC-5 (CCL-171) or WI-38 (CCL-75), HeLa T ⁺
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.

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 μ L of thawed stock culture.

10.4.2.3 Incubate for three days at $35 \pm 2^\circ\text{C}$ (should yield about 10^8 CFU/mL).

10.4.2.4 Centrifuge suspension at 5000 \times g for 15 min and resuspend pellet in $\frac{1}{10}$ initial volume of TPB.

10.4.2.5 Refer to Section 9 for the soil load.

10.5 *Trichophyton mentagrophytes*

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 $29 \pm 2^\circ\text{C}$.

10.5.1.10 Hemocytometer to count fungal conidia.

10.5.2 *Method:*

10.5.2.1 Streak a loopful (10 μ L) 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 $29 \pm 2^\circ\text{C}$ 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 1×10^7 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 $4 \pm 2^\circ\text{C}$. At three-month intervals, inoculate a fresh agar plate and incubate plate for ten days at $29 \pm 2^\circ\text{C}$.

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 μ m filter in it.

10.6.1.7 Incubator set at $35 \pm 2^\circ\text{C}$.

10.6.1.8 Black, gridded membrane filters 47 mm in diameter (0.45 μ m 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 $35 \pm 2^\circ\text{C}$ 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 \times g 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 $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$.

10.7.1.5 Incubator set at $35 \pm 2^\circ\text{C}$.

10.7.1.6 Orbital platform shaker.

10.7.2 *Method:*

10.7.2.1 To 99 mL of $\frac{1}{10}$ Columbia broth add 1 mL of 10 mM $\text{MnSO}_4 \cdot 4 \cdot \text{H}_2\text{O}$ solution.

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

10.7.2.3 Incubate at $35 \pm 2^\circ\text{C}$ 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 \times g 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 $\frac{1}{10}$ 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 $29 \pm 2^\circ\text{C}$.

10.8.1.5 *Method:*

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

10.8.1.7 Incubate at $29 \pm 2^\circ\text{C}$ for five days (should produce approximately 10^8 viable spores/mL).