Designation: E 2111 – 00

Standard Quantitative Carrier Test Method To Evaluate the Bactericidal, Fungicidal, Mycobactericidal and Sporicidal Potencies of Liquid Chemical Germicides[1]

This standard is issued under the fixed designation E 2111; 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 need for better tests to assess the germicidal activity of chemicals has been recognized **(1)**[2] and several investigations conducted in the past decade have been aimed at developing simpler and fully quantitative tests while attempting to design protocols suitable for working with a wide variety of microorganisms **(2)**. The method described here uses glass vials as carriers and the same basic set of materials and procedures can be used to test the potency of liquid chemical germicides against vegetative bacteria, fungi, mycobacteria and bacterial spores. It is not appropriate, however, for working with viruses because of the relatively high levels of eluate dilutions required and the need for membrane filtration. Further evaluation of products under more stringent test conditions may be necessary for their registration. Performance standards for the categories of products to be tested and the specific types of organism(s) to be used may also vary depending on the regulatory agency.

1. Scope

1.1 This test method is designed for use in product development and for the generation of product potency data. The loading of each carrier with a known volume of the test organism suspension is possible. The incorporation of control carriers also allows for an assessment of the load of viable test organism on the test carriers.

1.2 This test method is designed to have survivors and also to be used with a performance standard. The surviving microorganisms on each test carrier are compared to the mean of no less than three control carriers to determine if the performance standard has been met. To allow proper statistical evaluation of 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. For example, if an arbitrary performance standard of 6-$\log_{10}$ reduction in the viability titer of the test organism is used, and an inoculum size of 10^7 CFU, then theoretically a maximum of ten survivors per carrier is permitted; however, because of experimental variability the exact target may need to be higher than 10^6 CFU/carrier, thus fewer survivors would be permitted.

1.3 This test method should be performed by persons with training in microbiology and in facilities designed and equipped for work with infectious agents at the appropriate biosafety level **(3)**.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

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

1.6 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).

2. Referenced Documents

2.1 *ASTM Standards:*

D 1129 Terminology Relating to Water[3]

D 1193 Specification for Reagent Grade Water[3]

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

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides 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 parentheses refer to the list of references at the end of this standard.

[3] *Annual Book of ASTM Standards*, Vol 11.01.

2.2 *CFR Standards:*
40 CFR, Part 160 [4]
21 CFR, Part 58 [4]

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 applied inoculum of 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 This is a fully quantitative carrier test method suitable for assessing the potency of liquid chemical germicides against vegetative bacteria, fungi, mycobacteria, as well as bacterial spores. It is designed primarily for testing formulations to be used on hard environmental surfaces and medical devices. This test method uses the flat inside bottom surface of glass vials as the carrier. Each vial receives 10 µL of the test organism with or without a soil load. The contamination of the inside surface of the carrier with microaerosols is avoided by the use of glass inserts. The inoculum is dried and exposed to 1 mL of the test germicide for the desired contact time at the recommended temperature; control carriers receive 1 mL of phosphate buffer instead. At the end of the contact time, 9 mL of a diluent, with or without a neutralizer, is added to the vial to dilute/neutralize the germicide and any inoculum adhering to the carrier surface is recovered using a magnetic stir bar with a threaded surface. The eluate is passed through a membrane filter, the carrier vial is then rinsed several times with eluate/diluent and the rinses also are passed through the same filter. The total rinse volume is about 100 mL. Control and test eluates requiring dilution to get countable colonies, are subjected first to a series of 10-fold dilutions and the material from suitable dilutions is passed separately through membrane filters. Each filter is placed on the agar surface of an appropriate recovery medium in a 100-mm diameter petri plate. The plates are held for the required period at the desired incubation temperature, colonies counted and $\log_{10}$ reductions in the viability titer of the test organism calculated.

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

5. Significance and Use

5.1 This test method is fully quantitative and it also avoids any loss of viable organisms through wash off. This makes it possible to produce statistically valid data using many fewer test and control carriers than other quantitative methods based on most probable numbers (MPN).

5.2 The design of the carriers makes it possible to place into each a precisely measured volume of the test suspension. The use of the threaded stir bars allows for efficient recovery of the inoculum even after its exposure for several hours to strong fixatives such as glutaraldehyde.

5.3 The membrane filtration step 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.

5.4 In the absence of a universal neutralizer, this test method uses a 10-fold dilution of the test product by phosphate buffer or saline immediately at the end of the contact time and filtration of the organism-product mixture through a membrane and the subsequent rinsing of the filter with several changes of the buffer or saline. This approach usually reduces germicide residues to non-inhibitory levels; however, the test protocol permits the addition of a specific neutralizer to the eluent/diluent, if required (See Practices E 1054).

5.5 This test can be performed with or without a soil load to determine the effect of such loading on germicide performance. The soil load developed for this test is a mixture of three types of proteins (high molecular weight proteins, low molecular weight peptides and mucous material) to represent the body secretions, excretions or other extraneous substances that chemical germicides may encounter under field conditions. It is suitable for working with the various test organisms included here. The components of the soil load are readily available and subject to much less variability than animal sera.

5.6 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. 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 $CaCO_3$.

6. General Equipment and Labware

6.1 *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)**.

6.2 *Incubator*—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.

6.3 *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.

6.4 *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.

6.5 *Membrane Filtration System for Capture of the Test Organisms*—Sterile 47-mm diameter membrane filters (0.22 µm or 0.45 µm pore diameter) and glass or metal holders for such filters are required.

6.6 *Environmental Chamber/Incubator*—To hold the carriers at the desired test temperature.

6.7 *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.

6.8 *Refrigerator*—A refrigerator at 4±2°C for storage of media, culture plates and reagents.

6.9 *Timer*—Any stop-watch that can be read in minutes and seconds.

6.10 *Hot Air Oven*—An oven at 60°C to dry clean and sterile glassware.

6.11 *Magnetic Stir Plate and Stir Bars*—Large enough for a 5-L beaker or Erlenmeyer flask for preparing culture media or other solutions.

6.12 *Positive Displacement Pipette*—A pipette and pipette tips that accurately can dispense 10-µL volumes for inoculation of carriers.

6.13 *Air Displacement Pipettes*—Eppendorf or equivalent, 100–1000 µL with disposable tips.

6.14 *Orbital Shaker*—For shaking the broth cultures of bacteria during their incubation.

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

6.16 *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; reusable or disposable glass pipettes capable of handling 10-mL, 5-mL, and 1-mL volumes; 25-mL test tubes with caps.

6.17 *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.

6.18 *Sterile Disposable Plastic Petri Dishes*, 100 mm × 15 mm.

6.19 *Forceps*, straight or curved, with smooth tips to handle membrane filters.

6.20 *Flat-Bottomed Glass Vials*, 20 mL, with regular and septate caps (Fig. 1a). Flat-bottomed glass vials may be manufactured such that the bottom of the vials is completely flat with no ridges.[5,6]

6.21 *Vials*, wide-mouth, glass, 25 mL, for use as dilution vials.

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

6.23 *Stir Bars with Threaded TFE-fluorocarbon Coated Surface*, to dislodge inoculum from the carriers surface. Stir bars may be manufactured according to Fig. 1b.[7,6]

6.24 *Magnet*, strong enough to hold the threaded stir bar in place in the glass carrier while the liquid is being poured out of it for membrane filtration.

6.25 *Aluminum Foil*, to wrap items to be sterilized.

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

6.27 *Glass Inserts*, to be placed inside the glass carriers during their inoculation with the test organism. Such inserts have been found to eliminate the deposition of microaerosols on the inside walls of the carriers. Glass inserts may be manufactured according to Fig. 1c.[8,6]

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

6.29 *Markers*, permanent labware marking pens.

6.30 *Sterile Polypropylene Centrifuge Tubes with Caps*, 50 mL.

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

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

6.33 *Hemocytometer*, for counting fungal conidia.

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

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 **(4)**. Other grades may be used **(5)**, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 *Absolute Alcohol*—In a 100-mL plastic or glass beaker for flame-sterilization of metallic forceps used to handle membrane filters.

7.3 *Phosphate Buffer*, prepared according to the formulation given in Ref **(6)**. Adjust buffer pH to 7.2

7.4 *Sterile Normal Saline (0.85 % NaCl)*—To be used as an eluent for the mycobacterium and the fungus.

7.5 *Test Germicide*—Prepared at its use-dilution and brought to the test temperature.

7.6 *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.

7.7 $MnSO_4H_2O$, added to Columbia broth to promote *B. subtilis* sporulation.

7.8 *Test Product Diluent*, for test products requiring dilution to obtain a use-dilution, water with a standard hardness of 400 ppm as $CaCO_3$ may be used as the diluent.

7.9 *Deionized Distilled Water (DDW)*, for making reagent solutions and media. For terminology and specifications for water to be used refer to Terminology D 1129 and Specification D 1193 under 2.1.

7.10 *Plates of Recovery Media*—Media must be prepared and sterilized according to manufacturer's instructions and then aseptically dispensed into culture plates.

[5] The sole source of supply of flat-bottomed vials (catalog #5260G) known to the committee at this time is Galaxy Environ. Products (Newfield, NJ).

[6] 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.

[7] The sole source of supply of stirbars known to the committee at this time is Engineering Department, Rehabilitation Centre, Ottawa, Ontario, Canada.

[8] The sole source of supply of glass inserts known to the committee at this time is Galaxy Environ. Products (Newfield, NJ) .

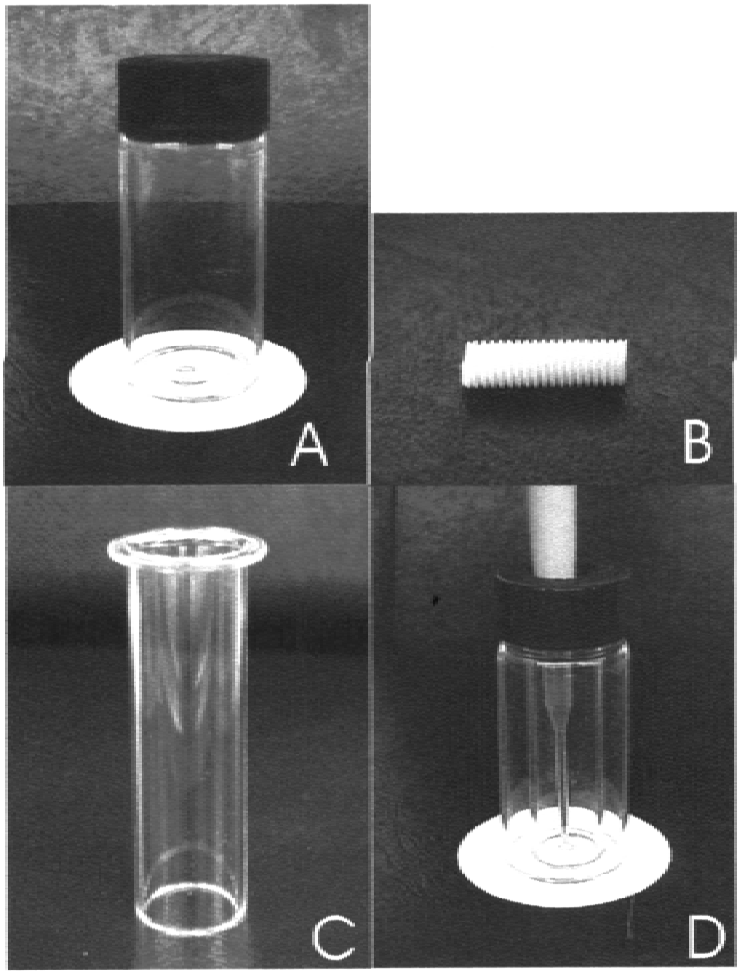


FIG. 1 Components of a Carrier for the Quantitative Carrier Test

A. Carrier Vial (28mm X 58mm); B. Magnetic Stir Bar (15mm X 4mm); C. Glass Insert (height 58 mm, diameter at bottom 16mm and diameter of flared end at the top 20mm); D. Carrier Inoculation

8. Carriers

8.1 *Preparation of the Carriers*—Place a clean glass insert inside each flat-bottomed vial and position the insert in place with the help of a septate cap loosely screwed on to the vial (see Fig. 1). Sterilize the required number of carriers, along with an equivalent number of regular caps for the carrier vials, in a container such that they can be stored without any contamination.

9. Soil Load

9.1 When a soil load is to be incorporated in the suspension of the test organism, it will consist of a mixture of the following stock solutions in phosphate buffer (pH 7.2):

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

9.1.2 Add 0.5 g of bovine serum albumin (BSA) to 10 mL of phosphate buffer.

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

9.1.4 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.

9.2 To obtain a 500 µL inoculum of the test organism, add to 340 µL of the microbial suspension 25 µL, 100 µL, and 35 µL of BSA, mucin and tryptone stock solutions, respectively.

NOTE 1—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. Preparing Inocula of Specific Types of Organisms

10.1 This method can be used with most species of vegetative and spore-forming bacteria as well as mycobacteria and fungi; however, Table 1 summarizes the species and strains of the test organisms most often used. The number of CFU/mL of each freshly prepared and properly homogenized microbial test suspension may be estimated spectrophotometrically, based on

TABLE 1 The Cultivation and Recovery of the Various Test Organisms to be Used in the Carrier Test

Organism (ATCC #)	Culture Medium	Recovery Medium
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	Tripticase soy agar; plates read after 48 h at 35±2°C
Conidia of *Trichophyton mentagrophytes* (9533)	Sabourauds' Dextrose Agar; incubation for 12 days at 29±2°C	Sabourauds' 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

a standard curve at a specific wavelength, but should be confirmed by membrane filtration.

10.2 The concentration of the test organism in the final suspension should be high enough to provide the test formulation with a challenge of no less than 10^6 CFU on each carrier. This is confirmed in each test by titrating the eluates from the control carriers.

10.3 *Staphylococcus aureus*:

10.3.1 *Materials*—Frozen stock of *S. aureus* (ATCC 6538).

10.3.2 Tryptose phosphate broth (TPB).

10.3.3 Trypticase soy agar (TSA).

NOTE 2—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.

10.3.4 *Method*—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.

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

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

10.3.7 Refer to Section 9 for the soil load.

10.4 *Pseudomonas aeruginosa*:

10.4.1 *Materials*—Frozen stock of *P. aeruginosa* (ATCC 15442).

10.4.2 TPB.

10.4.3 TSA.

10.4.4 *Method*—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.

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

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

10.4.4.3 Concentrate suspension by centrifugation and by resuspending the pellet in 1/10 the initial volume of TPB.

10.4.4.4 Refer to Section 9 for the soil load.

10.5 *Trichophyton mentagrophytes*:

10.5.1 *Materials*—Stock culture of *T. mentagrophytes* (ATCC #9533).

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

10.5.1.2 Sterile stainless steel spatula.

10.5.1.3 Sterile normal saline.

10.5.1.4 250-mL flask with glass beads (sterile).

10.5.1.5 Sterile absorbent cotton.

10.5.1.6 Sterile 150-mL glass beaker.

10.5.1.7 Bunsen burner.

10.5.1.8 Incubator set at 29±2°C.

10.5.1.9 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 centre of each of four Sabourauds' Dextrose Agar plates.

10.5.2.2 Incubate plates at 28°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 25–50 mL sterile saline (0.85 % NaCl) 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 Store at 2–10°C for up to four weeks for use in preparing test suspension of conidia for disinfection experiments.

10.5.2.9 Maintain stock culture of fungus on a Sabourauds' 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—M. terrae* (ATCC 15755) frozen stock.

10.6.1.1 Sterile deionized distilled water.

10.6.1.2 Sterile normal saline.

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 in each of four culture flasks.

10.6.2.2 Add 500 µL 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 1500 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 suspension in a bijoux bottle with ten glass beads (5-mm in diameter) 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*—Frozen stock of *B. subtilis* (ATCC 19659).

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

10.7.1.2 TSA.

10.7.1.3 Sterile 10 mM $MnSO_4.4\ H_2O$.

10.7.1.4 Incubator set at 35±2°C.

10.7.1.5 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 $MnSO_4.4\ H_2O$ 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 1000 xg 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*—Frozen stock of *C. sporogenes* (ATCC 7955).

10.8.2 Sterile full-strength Columbia broth.

10.8.3 Fastidious anaerobic agar (FAA).

10.8.4 Anaerobic incubator set at 29±2°C

10.8.5 *Method:*

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

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

10.8.8 Wash spore suspension three times by centrifuging it at 1500 xg 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.

10.8.9 Refer to Section 9 for the soil load.

11. Carrier Test

11.1 *Inoculation of the Carriers*—Wearing sterile gloves, gently tighten the septate caps on the carriers such that insert is positioned at the center of the bottom of the vial.

NOTE 3—The septate cap must not be screwed on too tight to avoid the touching and grinding of the narrow end of the insert on the inside bottom surface of the carrier vial.

11.1.1 Vortex the test suspension to evenly distribute cells/spores in it. Withdraw 10 µL of the suspension with a positive displacement pipette and place it onto the inside bottom surface of each carrier (Fig. 1d). For consistency, the same pipette tip can be used throughout the inoculation of a batch of carriers. Make sure that the inoculum does not touch the walls of the insert.

11.1.2 Allow the inoculum to 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).

11.1.3 Observe the dried inoculum on each carrier and discard any carrier in which the inoculum has touched the insert.

11.1.4 Wearing sterile gloves, remove the septate caps and inserts carefully, placing them in a bucket for subsequent decontamination and cleaning. Replace the septate caps with sterile regular caps and tighten.

11.1.5 These carriers are now ready for the test procedure.

11.2 *Exposure of the Organism(s) to the Formulation Under Test*—The number of test carriers to be used in each run is 10; however, in preliminary tests during product development only 3–5 carriers may be sufficient to assess the potency of experimental formulation(s) against the test organism(s).

11.2.1 In each carrier place 1 mL of test germicide over the dried inoculum and hold the carriers at the desired temperature for the desired contact period. At the end of the exposure time, aseptically place a sterile threaded stir bar in each carrier and

TABLE 2 Drying of the Test Organism Inoculated on the Carrier Surface

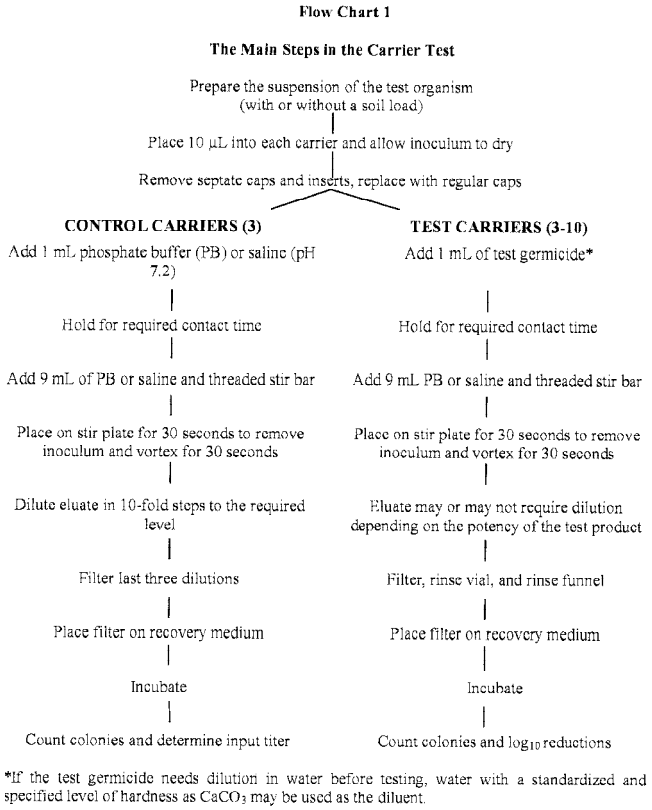
Test Organism	Drying of the Inoculum (at room temperature)
S. aureus	1 h in the open and one in a desiccator
P. aeruginosa	1 h in the open and one in a desiccator
Conidia of *T. mentagrophytes*	1 h in the open followed by overnight drying in a desiccator
M. terrae	1 h in the open followed by overnight drying in a desiccator
Spores of *B. subtilis*	1 h in the open followed by overnight drying in a desiccator
Spores of *C. sporogenes*	1 h in the open followed by overnight drying in a desiccator

immediately add 9 mL of sterile phosphate buffer or saline, with or without a neutralizer, to neutralize/dilute the germicide and arrest its activity.

11.2.2 Place the carrier on a magnetic stir plate and stir the contents of the carrier for 30 s to ensure the removal and resuspension of the inoculum from the bottom of the carrier. Vortex for 30 s.

11.2.3 Using a magnet to hold the stir bar in place, pour the contents of the vial in to the membrane filter holder. Rinse the carrier vial with 20 mL of phosphate buffer, vortex, and filter the rinse. Repeat rinse two more times. Rinse the sides of the funnel unit with at least 40 mL of phosphate buffer or 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.

NOTE 4—Presence of large numbers of colonies on the membrane filters from the test vials 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 the $\log_{10}$ reduction in viability by the test product, ten-fold dilutions of the eluate may be necessary (flow chart 1).



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

11.3.1 Instead of the test formulation, add 1 mL of sterile phosphate buffer or saline to each control carrier. The contact time and temperature for the control carriers must be the same as that for the test carriers.

11.3.2 At the end of the contact time, add 9 mL of sterile phosphate buffer or saline and a sterile threaded stir bar to each control carrier. Place the carrier on a magnetic stir plate for one minute to remove the inoculum from the bottom of the carrier. Vortex the carrier for approximately 30 s, or until any visible clumps have been broken.

11.4 *Dilution of the Eluates*:

11.4.1 The extent to which the eluates from the control carriers are to be diluted will depend on the number of viable cells in the inoculum and it will be necessary to determine the dilution range before hand to generate countable numbers of CFU for an accurate measurement of the challenge titer. Similarly, eluates from test carriers may require dilution to permit the calculation of $\log_{10}$ in the viability titer after exposure of the target organism(s) to the test formulation.

11.4.2 Make ten-fold dilutions of the eluates from control and test carriers. Pass the material from each dilution through separate membrane filters. Rinse the vial by adding 20 mL of phosphate buffer or saline, vortex, and filter the rinse through the same filter. Repeat the procedure two more times. Rinse the sides of the funnel unit with approximately 40 mL of phosphate buffer or saline. Aseptically transfer the filter to the recovery medium.

NOTE 5—Separate membrane filters, but the same filtration unit, can be used for processing all dilutions for a given carrier starting with the most dilute sample first.

11.4.3 Incubate the plates of the recovery medium at the required temperature for the desired length of time (see Table 1). Count the colonies and calculate the $\log_{10}$ reductions obtained.

12. Precision and Bias

12.1 *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. A collaborative study of 15 laboratories also has been carried out to determine the reproducibility of the data for the sporicidal activity of several blinded test samples. The carriers as well as the spore suspensions of *Bacillus subtilis* were provided to the participating laboratories, which tested the germicide samples against the spores without any soil load in the dried inocula. The test itself contributed only 5 % to the variability observed.

12.2 Other researchers have used the method for studies of germicidal activity **(7, 8)**.

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

NOTE 6—The development of this test method was made possible with financial assistance from the Antimicrobials Division of the U.S. Environmental Protection Agency.

13. Keywords

13.1 *Bacillus subtilis spores*; bactericides; chemical germicides; *Clostridium sporogenes spores*; eluate; eluent; environmental surfaces; fungicides; $\log_{10}$ reductions; medical devices; membrane filtration; mycobactericides; *Mycobacterium terrae*; *Pseudomonas aeruginosa*; quantitative carrier test; soil load; sporicides; *Staphylococcus aureus*; standard hard water; surrogate; *Trichophyton mentagrophytes conidia*

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