~~Designation:E2111–05~~ Designation: E2111 – 12

Standard Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal, and Sporicidal Potencies of Liquid ~~Chemical Microbicides~~Chemicals[1]

This standard is issued under the fixed designation E2111; 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 microbicidal activity of chemicals was recognized **(1)** [2] and several simpler and quantitative test methods have been developed for working with a wide variety of microorganisms **(2)**. The test method described here uses glass vials as carriers; the same basic set of materials and procedures can be used to test the potency of liquid microbicides against vegetative bacteria, fungi, mycobacteria, and bacterial spores. However, the test method is not appropriate for use 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. This test method permits the loading of each carrier with a known volume of the test organism. The incorporation of controls can also determine the initial load of colony forming units (CFU) of organisms on the test carriers and any loss in CFU after the mandatory drying of the inoculum.

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 In this test method, SI units are used for all applications, except for distance, in which case inches are used and SI 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~~CFR Part 160 for EPA submissions and 21 ~~CFR, Part 58~~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.*

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[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

~~Current edition approved Nov. 1, 2005. Published December 2005. Originally approved in 2000. Last previous edition approved in 2000 as E2111–00. DOI: 10.1520/E2111–05.~~

Current edition approved May 15, 2012. Published June 2012. Originally approved in 2000. Last previous edition approved in 2005 as E2111 – 05. DOI: 10.1520/E2111–12.

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

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~~ Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E2197 Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *CFR Standards:*[4]

~~40 CFR,~~40 CFR Part 160

21 ~~CFR,~~CFR Part 58

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

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

3.1.2 *eluate*, *n*—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*—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. (Refer to ~~Practices~~Test Methods E1054 ~~in 2.1~~ for further details

3.1.4.1 *Discussion*—This process may be achieved by dilution of the organism/test formulation mixture or by adding to it one or more chemical neutralizers, or both.

3.1.5 *soil load*, *n*—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*—formulation that incorporates antimicrobial ingredients.

3.1.7 *test organism*, *n*—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 chemicals 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 microbicide for the desired contact time at the recommended temperature; control carriers receive 1 mL of normal saline instead. At the end of the contact time, 9 mL of an eluent without or with a neutralizer, is added to the vial to dilute/neutralize the microbicide 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 eluent/diluent and the rinses are also passed through the same filter. The total rinse volume is no less than 100 mL. Control and test eluates requiring dilution to get countable colonies are first subjected to a series of tenfold 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.

NOTE 1—Do not soak the magnetic stir bars in ethanol or other solvents for decontamination as this may damage the sealant on them.

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~~off, making 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.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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

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.

~~5.4This test can be performed with or without a soil load to determine the effect of such loading on microbicide 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 microbicides 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.~~

~~5.5Since 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 $CaCO_3$.~~

5.4 This test can be performed with or without a soil load to determine the effect of such loading on microbicide performance. Consult the target regulatory agency on the need, type(s), and acceptable level(s) of soil load prior to testing. One type of soil load (Quantitative Disk Carrier Test Method E2197) to consider 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 microbicides 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.

5.5 If distilled water or other diluent is not to be specified on the product label, the diluent for the test substance is assumed to be tap water. Since the quality of tap water varies considerably both geographically and temporally, this test method incorporates the use of water with a specified and documented level of hardness to prepare use-dilutions of test substance that require dilution in water before use. Consult the target regulatory agency regarding the use and level of water hardness prior to testing.

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- or 0.45-µm pore diameter) and glass, metal, or plastic holders for such filters are required.~~ —Sterile 47-mm diameter sterilizing membrane filters and glass, metal, or plastic holders for such filters are required. Membranes made from polyethersulfone (PES) are recommended. Filter membranes with a pore diameter of 0.22 µm must be used when working with bacterial spores.

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, plates, and reagents.

6.9 *Timer*—Any stopwatch that can be read in minutes and seconds.

6.10 *Hot Air Oven*—An oven at 60°C to dry and sterile clean 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 to 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*—One-liter 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; 100 mL and 5 L beakers, reusable or disposable glass pipettes capable of handling 10-, 5-, and 1-mL volumes; and 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 by 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

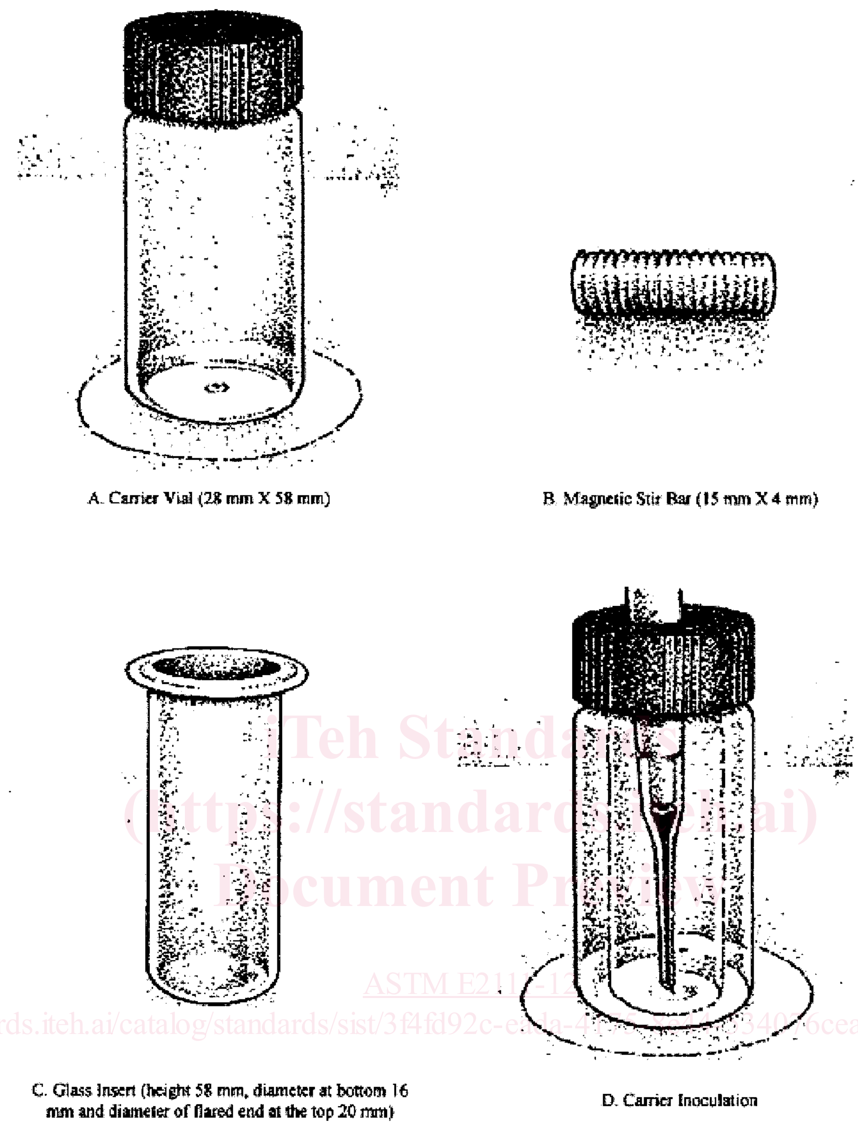

FIG. 1 Components of a Carrier for the Quantitative Carrier Test

such that the bottom of the vials is completely flat with no ridges.~~,~~[5]

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

6.22 *Desiccator*, recommended size is 25 cm wide by 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.[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.

~~[5] The sole source of supply of flat-bottomed vials (catalog #5260G) known to the committee at this time is Galaxy Environ. Products, P.O. Box 238, 7 Greenwood Ave., Newfield, NJ 08344.~~

[5] The sole source of supply of the apparatus (flat-bottomed vials (catalog #5260G)) known to the committee at this time is Galaxy Environ. Products, P.O. Box 238, 7 Greenwood Ave., Newfield, NJ 08344. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

~~[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.~~

[6] The sole source of supply of stir bars known to the committee at this time is Engineering Department, Rehabilitation Centre, 505 Smyth Rd., Ottawa, ON, Canada K1H 8M2; phone: 613-737-7350, ext. 75320. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

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 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. 1~~C.~~[6] C.[7]

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

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.

6.35 *Bunsen Burner*, for aseptic technique

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 *Normal Saline (0.85 % NaCl; pH 7.2)*—To be used as an eluent and control fluid.

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

7.5 *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.

7.6 $MnSO_4$ H_2O, added to Columbia broth to promote the *B. subtilis* sporulation.

7.7 *Test Product Diluent*, water with a standard hardness of ~~400~~at least 300 ppm as $CaCO_3$ may be used as the diluent, for test products requiring dilution in water to obtain a use-dilution.

7.8 *Deionized Distilled Water (DDW)*, for making reagent solutions and media. For terminology and specifications for water to be used refer to Terminology D1129 and Specification D1193 under 2.1.

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

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. 1D). 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

~~9.1When 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 saline (pH 7.2):~~

~~9.1.1Add 0.5 g of tryptone to 10 mL of saline.~~

9.1 Refer to 5.4 with regards to the addition of a soil load. In case a soil load is required, the following mixture is an example of a generic soil load. When a soil load is required in the testing, mix it with the suspension of the test organism. Prepare the stock solutions of the soil load components in saline (pH 7.2) as follows:

9.1.1 Add 0.5 g of Tryptone or yeast extract to 10 mL of saline.

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

9.1.3 Add 0.04 g of bovine mucin to 10 mL of saline.

9.1.4 Prepare the solutions separately and sterilize by passage through a 0.22 µm pore diameter membrane ~~filter, aliquot and store~~filter. Aliquot in volumes for single-use in vials for storage 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, 100, and 35 µL of BSA, mucin, and tryptone stock solutions, respectively.

NOTE 2—Animal sera, often used as a soil load, vary widely in their composition and may also contain microbial inhibitors. The soil load mixture given

~~[7] The sole source of supply of stir bars known to the committee at this time is Engineering Department, Rehabilitation Centre, 505 Smyth Rd., Ottawa, ON, Canada K1H 8M2; phone: 613-737-7350, ext. 75320.~~

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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 test method can be used with most species of vegetative and spore-forming bacteria as well as mycobacteria and fungi; however, Table 1Appendix X1 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 a standard curve at a specific wavelength, but should be confirmed by membrane filtration.

10.2The concentration of the test organism in the dried inoculum on each carrier must be equal to or higher than the product performance criterion to be met. This is confirmed in each test by titrating the eluates from the control carriers.

10.2 In general, this number should not be more than 10× the defined performance standard. This should be confirmed in each test by determining the numbers of viable organisms on the control carriers

NOTE 3—TSA and TSB, which are based on soybean-casein digests, were used in the development of the test method described here. Other media with similar formulations may be used instead.

10.3*Staphylococcus aureus*:

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

10.3.2Tryptose soy broth (TSB).

10.3.3Trypticase soy agar (TSA).

10.3.4*Method*—Prepare 100 mL of TSB according to the manufacturer's instructions and distribute aliquots of approximately 10 mL into the appropriate number of test tubes. Sterilize as per manufacturer's instructions.

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

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

10.3.7Refer to Section 9 for the soil load.

10.4*Pseudomonas aeruginosa*:

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

10.4.2TSB.

10.4.3TSA.

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

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

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

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

10.4.4.4Refer to Section 9 for the soil load.

10.5*Trichophyton mentagrophytes* :

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

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

10.5.1.2Sterile stainless steel spatula.

10.5.1.3Sterile normal saline.

10.5.1.4250-mL flask with glass beads (sterile).

10.5.1.5Sterile absorbent cotton.

10.5.1.6Sterile 150-mL glass beaker.

10.5.1.7Bunsen burner.

10.5.1.8Incubator set at 29 ± 2°C.

10.5.1.9Hemocytometer to count fungal conidia.

10.5.2*Method*:

10.5.2.1Streak a loopful (10 µL) of thawed stock culture of *T. mentagrophytes* at the center of each of four SDA plates.

10.5.2.2Incubate plates at 29 ± 2°C for not less than 10 days and not more than 15 days.

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

10.5.2.4Transfer to 250-mL flask containing 25- to 50-mL sterile saline (0.85% NaCl) with glass beads; shake flask vigorously enough to break off the conidia from the hyphae.

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

10.5.2.6Estimate density of conidial suspension by counting in hemocytometer.

10.5.2.7Standardize suspension as needed by diluting it with sterile saline so that it contains about 1×10^7 conidia/mL.

10.5.2.8Store at 2 to 10°C for up to four weeks in preparing test suspension of conidia for disinfection experiments.

10.5.2.9Maintain stock culture of fungus on SDA plate at 4 ± 2°C. At three-month intervals, inoculate a fresh agar plate and incubate plate for ten days at 29 ± 2°C.

10.5.2.10Refer to Section 9 for the soil load.

10.6*Mycobacterium terrae*:

10.6.1*Materials*—Frozen stock*M. terrae* (ATCC 15755).