



Designation: E 2111 – 00

Standard Quantitative Carrier Test Method To Evaluate the Bactericidal, Fungicidal, Mycobactericidal and Sporocidal Potencies of Liquid Chemical Germicides¹

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:*³

D 1129 Terminology Relating to Water

D 1193 Specification for Reagent Grade Water

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 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.

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

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.

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.

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

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 $-20 \pm 2^\circ\text{C}$ 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 $4 \pm 2^\circ\text{C}$ 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.⁹

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 *MnSO₄H₂O*, 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 CaCO₃ may be used as the diluent.

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).

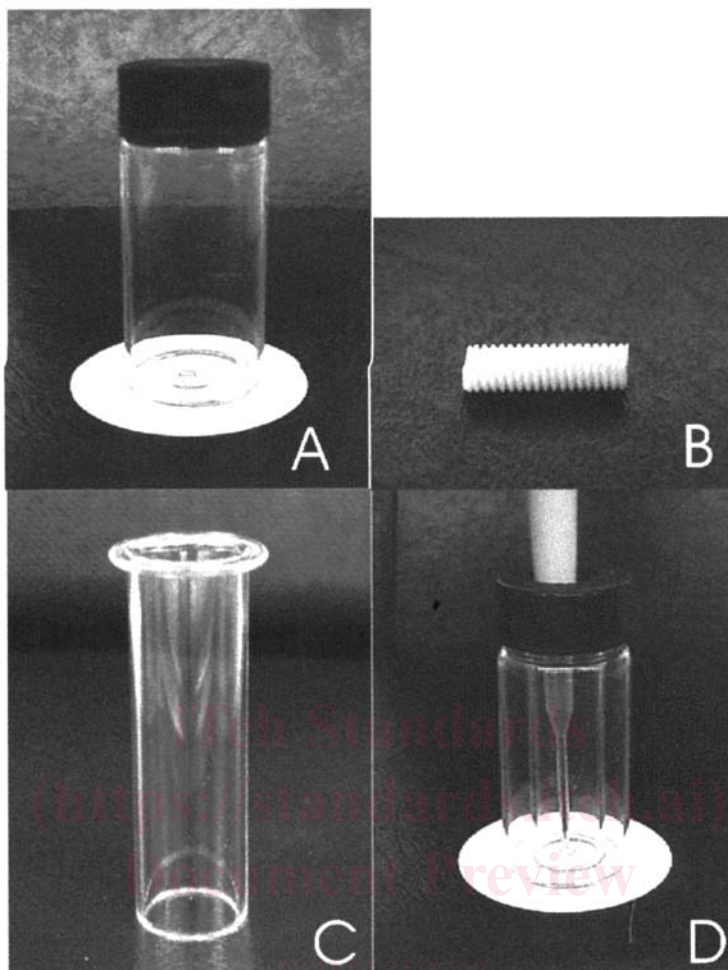


FIG. 1 Components of a Carrier for the Quantitative Carrier Test

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

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.

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 $4\pm 2^\circ\text{C}$ or $-20\pm 2^\circ\text{C}$.

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