Designation: E2197 – 17ε1

Standard Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals[1]

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.

ε[1] NOTE—Sections 8.1 and 11.8 were editorially corrected in January 2018.

INTRODUCTION

The quantitative test method described here uses disks of stainless steel (1 cm in diameter) as carriers. It employs the same basic set of materials and procedures to assess the ability of liquid chemicals to inactivate vegetative bacteria, viruses, fungi, mycobacteria, and bacterial spores (**1-7**).[2] Performance standards for test substances, the level of water hardness, the type and level of a soil load, the test organism(s), and other test conditions may vary depending on the target regulatory agency. This basic test can also be adapted for use with other carrier materials of similar dimensions.

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

1. Scope

1.1 This test method is designed to evaluate the ability of test substances to inactivate vegetative bacteria, viruses, fungi, mycobacteria, and bacterial spores (**1-7**) on disk carriers of brushed stainless steel that represent hard, nonporous environmental surfaces and medical devices. It is also designed to have survivors that can be compared to the 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 number of viable organisms in the test inoculum should be sufficiently high to take into account both the performance standard and the experimental variations in the results.

1.2 The test protocol does not include any wiping or rubbing action. It is, therefore, not designed for testing 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 (**8**).

1.4 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).

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

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

A967/A967M Specification for Chemical Passivation Treatments for Stainless Steel Parts

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

[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.

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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.

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *CFR Standard:*[4]

21 CFR, Part 58 Laboratory Practice for Nonclinical Laboratory Studies

40 CFR, Part 160 Good Laboratory Practice Standards

2.3 *CEN Standard:*[5]

EN 10088-2 1J/2J Stainless steels - Part 2: Technical delivery conditions for sheet/plate and strip of corrosion resisting steels for general purposes

3. Terminology

3.1 *Definitions*—For definitions of general terms used in this test method, refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

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

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

3.2.3 *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.

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

3.2.5 *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.

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

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

4. Summary of Test Method

4.1 Each disk (1 cm in diameter) receives 10 µL of the test organism with a soil load. The inoculum is dried, and then the disk is placed on the inside bottom surface of a sterile plastic vial prior to contact with 50 µL of the use-dilution of test substance. The contact time and temperature may vary as required. Control carriers receive 50 µL of a fluid harmless to the test organism(s) and its host cells, if any, but are otherwise treated in the same way as test carriers.

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

4.3 For tests with viruses, appropriate dilutions of the eluate are inoculated into suitable cell cultures, the cultures are examined for cytopathology/infectious foci, which are estimated as the most probable number (MPN) or counted as foci or plaques, and $\log_{10}$ are calculated.

5. Significance and Use

5.1 The design of this test eliminates 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 MPN estimates.

5.2 The stringency in the test is provided by the use of a soil load, the microtopography of the brushed stainless steel carrier surface, and the smaller ratio of test substance to surface area typical for many disinfectant applications. Thus, the test substance being assessed is presented with a reasonable challenge while allowing for efficient recovery of the test organisms from the inoculated carriers. The metal disks 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 a precisely measured volume of the test organism (10 µL) as well as the control fluid or test substance (50 µL).

5.4 The inoculum is placed at the center of each disk whereas the volumes of the test substance covers nearly the entire disk surface, thus virtually eliminating the risk of any organisms remaining unexposed.

5.5 In all tests, other than those against viruses, the addition of 10 mL of an eluent/diluent gives a 1:200 dilution of the test substance immediately at the end of the contact time. While this step in itself may be sufficient to arrest the microbicidal activity of most actives, the test protocol permits the addition of a specific neutralizer to the eluent/diluent, if required. Except for viruses, 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 saline also reduces the risk of carrying any inhibitory residues over to the recovery medium. Validation of the process of neutralization of the test substance is required by challenge with low numbers of the test organism.

5.6 In tests against viruses, addition of 1 mL of buffer at the end of the contact time achieves a 1:20 dilution of the test substance 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 substance 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 (See Appendix).

NOTE 1—In 5.5 and 5.6, volumes of 10 mL and 1 mL are recommended instead of 9.95 mL and 950 µL, respectively, for ease of dispensing the eluent.

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

[5] Available from European Committee for Standardization (CEN), Avenue Marnix 17, B-1000, Brussels, Belgium, http://www.cen.eu.

5.7 The soil load 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 body secretions, excretions, or other extraneous substances that microbicidal chemicals 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.

5.8 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. While water with a hardness of at least 300 ppm as $CaCO_3$ is recommended consult local regulations regarding use of hard water prior to testing.

5.9 The Annex contains a list of those organisms that are often used in assessing the microbicidal activities of disinfectants for use on environmental surfaces or medical devices. Culture conditions for each organism are also included in the Annex. Depending on the label claim(s) desired and the requirements of the target regulatory agency, one or more of the organisms listed may be selected for the testing. If organisms other than those listed are to be used (for example, in the dairy or brewing industries), a clear justification must be provided and details of the culture media and growth 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,* (see Note 2) plastic cell culture flasks of 25- and 75-cm^2 capacity for culturing cells and for preparing virus pools; 12-well or 96-well plastic plates for titrating virus infectivity.

NOTE 2—Plastic culture ware may be purchased from most laboratory supply houses.

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

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

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

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. The procedures for the proper maintenance and use of such cabinets are given in Ref (**8**).

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

6.20 *Markers,* for permanent marking of labware.

6.21 *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.

6.22 *pH Meter,* to measure pH of buffers, eluents, and test formulations.

6.23 *Microwave Oven,* to melt agar overlays.

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

6.25 *Orbital Shaker,* for shaking the broth cultures of *Bacillus 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 accurately dispense 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.