Designation: ~~E3218 – 19~~ E3218 – 21

Standard Test Method for Quantitative Method for Testing Antimicrobial Agents against Spores of *C. difficile* on Hard, Nonporous Surfaces[1]

This standard is issued under the fixed designation E3218; 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.

1. ~~Scope~~ Scope*

1.1 This test method covers a standardized approach to quantitatively determine the effectiveness of antimicrobial chemicals in treating hard, non-porous surfaces contaminated with spores of *C. difficile* (ATCC 43598) grown in accordance with Practice E2839.

1.2 This test method is based on principles established for Test Method E2197 and an Organisation for Economic Co-operation and Development Guidance Document.[2]

1.3 Training in basic microbiology and aseptic technique are required to perform this assay.

1.4 *Units*—The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *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.*

1.6 *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 Specification for Chemical Passivation Treatments for Stainless Steel Parts

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

E2839 Practice for Production and Storage of Spores of ***C. difficile*** for Use in Efficacy Evaluation of Antimicrobial Agents

2.2 *Other Standards*[2]

OECD Guidance Document Quantitative Method for Evaluating Bactericidal Activity of Microbicides used on Hard Non-Porous Surfaces. Dated June 21, 2013.

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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.

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[2] Available from the Organisation for Economic Co-operation (OECD) 2, rue André Pascal 75775 Paris Cedex 16, France, www.oecd.org

[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.

***A Summary of Changes section appears at the end of this standard**

3. Terminology

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

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *frozen spore suspension, n*—A preparation of bacterial endospores that is maintained at ~~-80 ± 5 °C.~~ -80 °C ± 5 °C.

3.2.1.1 *Discussion*—
For the purposes of this test method, the definition applies to spore suspensions of *C. difficile* that have been prepared and qualified in accordance with Practice E2839.

3.2.1.2 *Discussion*—
Spore suspensions of *C. difficile* used in this test method may be stored for up to 90 days under the conditions provided in the definition.

3.2.2 *final test suspension, n*—thawed frozen spore suspension (3.2.1) including the addition of a soil load.

3.2.3 *test system control, n*—a solution of ~~1500 ± 150~~ 1500 ppm ± 150 ppm laboratory-grade sodium hypochlorite (NaOCl) used to validate each efficacy test.

3.3 *Acronyms:*

AISI = American Iron and Steel Institute
CFU = Colony-forming unit

4. Summary of Test Method

4.1 The method uses disks (1 cm in diameter) of brushed stainless steel to represent hard, non-porous surfaces.

4.2 Each disk receives 10 µL of the final test suspension.

4.3 The final test suspension is dried and exposed to 50 µL of the test chemical (treated carriers) or 50 µL of a control fluid (control carriers). The contact time is allowed to elapse and an appropriate neutralizer is added at the end of the contact time.

4.4 The neutralized carriers are vortexed and the resulting suspension is serially diluted and filtered to determine the presence of spores.

4.5 Based on mean $\log_{10}$ density values, the $\log_{10}$ reduction (LR) in the viability of the test organism on treated carriers is calculated in relation to the viability count on the control carriers.

4.6 With each efficacy test, three inoculated carriers are exposed to a treatment consisting of 50 µL of ~~1500 ± 150~~ 1500 ppm ± 150 ppm NaOCl. The mean LR (<3.0) in the viability of the spores on test system control carriers ensures the validity of the data.

5. Significance and Use

5.1 The test method was designed to determine the LR in spores on a hard, non-porous surface after exposure to a test chemical in a closed system.

5.2 Each test includes three control carriers (exposed to phosphate buffered saline with Tween-80), three test system control carriers (exposed to ~~1500 ± 150~~ 1500 ppm ± 150 ppm sodium hypochlorite), and ten treated carriers (per test chemical/concentration/contact time combination).

6. Interferences

6.1 *Clostridioides difficile* (ATCC 43598), formerly known as *Clostridium difficile*, is an obligate anaerobe and must be incubated under strict anaerobic conditions. *C. difficile* will not grow in the presence of oxygen. Verification of anaerobic conditions is required.

7. Apparatus

7.1 Carriers, flat disks (1 cm in diameter) made ~~from approximately 0.8 mm thick sheets of brushed and magnetized stainless steel (AISI 430).~~of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side.

NOTE 1—The precision and bias statement provided in Section 12 was based on testing performed using AISI 430 stainless steel carriers; the precision statistics summarized in Section 12 do not apply for testing performed using other alloys.

~~7.1.1 Disks (1 cm in diameter) made from approximately 0.8 mm thick sheets of brushed stainless steel (AISI 304) may be used instead of 430 stainless steel carriers for testing oxidative chemistries such as peracetic acid, peroxides, or other oxidizing chemistries.~~

~~NOTE 1—The precision and bias statement provided in Section 12 was based on testing performed using AISI 430 stainless steel carriers; the precision statistics summarized in Section 12 do not apply for testing performed using other alloys.~~

7.1.1 The top of the carrier is brushed; only the top is visually screened and inoculated. Carriers are single-use only. See Annex A1 for carrier specifications.

7.2 *Calibrated 10 µL positive displacement pipette with corresponding 10 µL tips,* for carrier inoculation.

7.3 *Calibrated micropipettes (for example, 200 µL) with 10-100 or 20-200 µL tips,* for preparing aliquots of soil and spores; for deposition of test chemical on carrier.

7.4 *Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes,* for rinsing vials and filters.

7.5 *Sterile forceps,* to pick up the carriers for placement in vials and to handle membrane filters.

7.6 *Filter paper,* 150 mm diameter, to line Petri plates.

~~7.7 *Magnet,* use to hold magnetized carriers in place in the vial while the liquid is being dispensed poured into filter unit.~~

7.7 *Polyethersulfone membrane filter (PES),* for recovery of test spores, 47 mm diameter and 0.2 µm pore size. Any filtration apparatus may be used including filtration units (reusable or disposable).

7.8 *Vials with lids (plastic or comparable),* sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), approximately 20 mL capacity, for holding inoculated carriers to be exposed to the test chemical and for accommodating neutralizer.

7.9 *Vortex mixer,* to vortex the fluid in the vials to ensure efficient recovery of the test organism.

7.10 *Certified timer,* readable in minutes and seconds.

7.11 *Desiccator,* (with gauge to measure vacuum) with fresh desiccant (for example, $CaCO_3$), for drying the inoculum on the carriers.

7.12 *Vacuum source,* in-house line or suitable vacuum pump (0.068 to 0.085 MPa) for drying carriers and for membrane filtration.

7.13 *Microscope,* with 10× eyepiece and 100× (oil) objective with phase contrast option. To examine spores.

7.14 *Anaerobic chamber,* supported by a gas mixture containing at least 5 % H_2 with the balance comprising any inert gas such as CO_2, N_2, or Ar; refer to chamber manufacturer's recommendations. Use to ensure anaerobic environment.

NOTE 2—An activated anaerobic jar or other chamber may be used according to manufacturer's instructions in place of the anaerobic chamber.

7.15 *Incubator,* use an incubator at 36 ± 1 °C inside an anaerobic chamber to support the growth of the organism. Alternatively, place anaerobic jars in an incubator at ~~36 ± 1 °C.~~36 °C ± 1 °C.

7.16 *Digital titrator kit,* to measure total chlorine and water hardness. Alternate titration methods may be used.

7.17 *Laboratory film or sterile bags (18 by 30 cm or equivalent),* to retain the moisture in plates during prolonged incubation in an anaerobic chamber.

8. Reagents and Materials

8.1 *Culture Media:*

8.1.1 *Recovery medium*—brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT).[4] For enumeration of spores, commercially available as pre-reduced.

8.2 *Reagents:*

8.2.1 *Water*—all references to water as diluent or reagent shall mean de-ionized water or water of equal purity.

8.2.2 *Phosphate buffered saline (PBS)*—for use as a rinsing agent and to prepare PBS containing 0.1 % (v⁄v) Tween-80 (PBS-T) and PBS-T with 0.1 % (w/v) sodium thiosulfate; adjust pH to 7.0 ± 0.5 if necessary.

8.2.3 *PBS containing 0.1% (v/v) Tween 80 (PBS-T)*—diluting reagent; adjust pH to 7.2 ± 0.2 if necessary.

8.2.4 *PBS-T with 0.1 % (w/v) sodium thiosulfate*—neutralizer for the test system control ~~(1500 ± 150~~(1500 ppm NaOCl ± 150 ppm NaOCl); PBS-T with sodium thiosulfate pH is 7.2 ± 0.2.

8.2.4.1 Confirm the effectiveness of PBS-T with 0.1 % (w/v) sodium thiosulfate as a neutralizer for ~~1500 ± 150~~1500 ppm ± 150 ppm NaOCl using the procedure in Annex A2.

8.2.5 *Neutralizer*—specific to disinfectant test chemical being evaluated as determined for effectiveness and toxicity according to Annex A2. Use a neutralizer containing 0.1 % (v/v) Tween 80 to reduce spore clumping.

8.2.5.1 Conduct neutralization testing to confirm the neutralizer's effectiveness in accordance with Annex A2.

8.2.6 *Soil load,* the standard soil load to be incorporated in the qualified spore suspension is a mixture of the following stock solutions:[2]

8.2.6.1 *Bovine serum albumin (BSA),* Add 0.5 g BSA (radio immunoassay (RIA) grade or equivalent) to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter to sterilize.

8.2.6.2 *Yeast extract,* Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter to sterilize.

8.2.6.3 *Mucin,* Add 0.04 g mucin (from bovine submaxillary gland or equivalent) to 10 mL of PBS, mix thoroughly until dissolved, and ~~autoclave (15 min at 121 °C).~~pass through a 0.2 µm pore diameter membrane filter.

[4] The sole source of supply of the BHIY-HT (Cat. No. AS-6463) known to the committee at this time is Anaerobe Systems, Morgan Hill, CA. 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,[1] which you may attend.

8.2.6.4 Aseptically aliquot soil stock solutions and store for up to one year at ~~-20 ± 5 °C.~~-20 °C ± 5 °C. The stock solutions of the soil load are single use only; do not refreeze once thawed.

NOTE 3—Intermittently vortex soil stock solutions while preparing aliquots.

NOTE 4—Other volumes of the stock solutions may be prepared following the same ratio.

8.2.7 *Test chemical,* antimicrobial test solution.

8.2.8 *Reagent grade sodium hypochlorite (NaOCl) with total chlorine ≥4%,* to prepare ~~1500 ± 150~~1500 ppm ± 150 ppm total chlorine for test system control.

8.2.9 *Tween-80 (polysorbate 80),* to make dilution blanks and neutralizers.

8.2.10 *Laboratory detergent (1 % solution),* to clean carriers.

9. Culture/Inoculum Preparation

9.1 Prepare spores of *C. difficile* (ATCC 43598) according to Practice E2839.

9.1.1 Spores may be stored at ~~-80 ± 5 °C~~-80 °C ± 5 °C for up to 90 days prior to use.

9.1.2 The mean $\log_{10}$ density (LD) of spores for control carriers is 6.0 to 7.0 spores/carrier, with each control carrier exhibiting a LD of 6.0 to 7.0.

10. Procedure

10.1 *Preparation and Sterilization of Carriers:*

10.1.1 Without magnification, visually check the brushed top surface of the carriers for abnormalities (for example, rust, chipping, atypical brushed striations) and discard if observed; refer to ~~A1.3~~A1.2 for examples of typical acceptable and unacceptable carriers.

10.1.2 Soak visually screened carriers in a suitable laboratory detergent solution free from any antimicrobial activity for 2 to 4 h to degrease and then rinse thoroughly in distilled or deionized water. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.

10.1.3 Using gloved hands or forceps, place up to 20 clean dry carriers on a piece of filter paper inside the bottom surface of a glass Petri dish (150 mm in diameter), ensure carriers were not damaged (scratched) during processing.

10.1.4 Cover the Petri dish with its lid and sterilize by autoclaving for 45 min at 121 °C on a gravity cycle.

NOTE 5—Alternative validated sterilization cycles may be used to sterilize carriers.

NOTE 6—Place Petri dish with carriers in autoclave pouch for sterilization.

10.1.5 Visually inspect carriers to ensure that they are dry following sterilization.

10.1.6 After sterilization, aseptically transfer carriers with forceps to sterile plastic Petri dishes without filter paper for inoculation.

10.2 *Final Test Suspension Preparation:*

10.2.1 Defrost a cryovial of the qualified spore suspension at room temperature. Each cryovial is single use only.

10.2.2 Vortex the thawed spore suspension for ~~45~~45 s to 60 s to resuspend the spores.

10.2.3 Add the spore suspension to the three-part soil load.

10.2.3.1 To obtain 500 µL of the final test suspension, vortex each component and combine the following (or appropriate ratio): 25 µL BSA stock, 35 µL yeast extract stock, 100 µL mucin stock, and 340 µL spore suspension.

10.3 *Carrier Inoculation:*

10.3.1 Following the combination of the spore suspension and the soil load, vortex-mix the final test suspension for approximately 10 s; use within 30 min for carrier inoculation.

10.3.2 For carrier inoculation:

10.3.2.1 Withdraw 10 µL of the final test suspension with a calibrated positive-displacement pipette (with a 10 µL pipette tip) and deposit the final test suspension in the center of each carrier.

10.3.2.2 Inoculate a sufficient number of carriers for testing (for example, ten carriers exposed per test chemical/concentration/contact time combination, three exposed to the test system control, three control carriers, plus extras (for example, three extra carriers)).

10.3.2.3 Vortex-mix the final test suspension for approximately 5 s after inoculating every 5 carriers.

10.3.2.4 When inoculating, avoid contact of pipette tip with the carrier; do not spread the final test suspension with the pipette tip.

10.3.2.5 The same pipette tip may be used to inoculate each batch of carriers.

10.3.2.6 Discard any inoculated carrier where the final test suspension has run over the edge of the carrier.

10.3.3 Dry the carriers inside a plastic Petri plate (up to 15 carriers/Petri plate) with the lid off in a biological safety cabinet (BSC) (up to 60 min or until the inoculum is visibly dry).

10.3.4 After the inoculum has dried, place the Petri plate without the lid in a desiccator connected to a vacuum line.

10.3.4.1 Cover the desiccator and make sure that it is properly sealed.

10.3.4.2 Continue drying the carriers (with the lid off the Petri plate) under vacuum maintained at 0.068 MPa to 0.085 MPa for ~~120 ± 5~~120 min ± 5 min at room temperature.

10.3.5 At the end of the drying period, turn off the vacuum, and cover the plate. Observe the dried inoculum on each carrier. Refer to Fig. 1 for an example of a typical dried carrier.

10.3.5.1 Discard any carrier on which the inoculum has dried near the edge of the carrier or has run off of the surface.

10.3.5.2 Use inoculated carriers immediately or store the inoculated carriers in the desiccator without vacuum.

10.3.5.3 Use dried carriers within 24 h of inoculation.

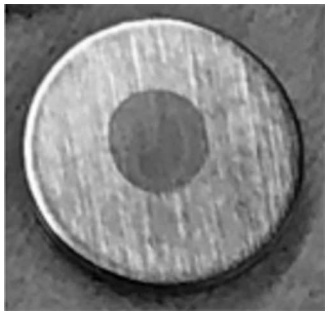

FIG. 1 Typical Dried Carrier Inoculated with 10 µL of the Final Test Suspension

10.4 *Prepare Test Chemical:*

10.4.1 When preparing the test chemical, ensure that the test chemical is adequately mixed. Use within 3 h of preparation or as specified in the manufacturer's instructions.

NOTE 7—Measuring error increases as delivery volume decreases. To minimize variability due to measuring error, a minimum of 1.0 mL or 1.0 g of concentrated test chemical should be used when preparing use-dilutions for testing. Use v/v dilutions for liquid test chemicals and w/v dilutions for solid test chemicals.

10.4.2 Evaluate the test chemical at room temperature ~~(22 ± 2 °C).~~(22 °C ± 2 °C). If necessary, place test chemical in water bath prior to use to equilibrate to the appropriate temperature for approximately 10 min. Record temperature.

10.5 *Efficacy Evaluation—Treated Carriers:*

10.5.1 Using sterile forceps, transfer each dried carrier with the inoculated side up to a flat-bottomed vial and cap the vial until treatment. Repeat until all carriers are transferred.

10.5.2 In a timed fashion at predetermined staggered intervals, deposit 50 µL of the test chemical (treatment) over the dried inoculum on the carriers, ensuring complete coverage of the inoculum.

10.5.2.1 Use a new tip for each carrier; do not touch the pipette tip to the carrier surface.

10.5.2.2 During testing, do not process carriers where the test substance runs off of the carrier; replace with new carrier(s) and vial(s) if this occurs.

10.5.2.3 Do not cap the vials.

10.5.3 Hold carriers at ~~22 ± 2 °C~~22 °C ± 2 °C for specified contact time.

10.5.4 Within ± 3 s of the end of the contact period, add 10 mL of neutralizer at room temperature to each vial in the specified order according to the predetermined staggered intervals.

10.5.5 Cap the vial and briefly vortex ~~(2~~(2 s to 3 s). The neutralized vial is the 10^0 dilution.

10.5.6 Following the neutralization of the entire set of carriers, vortex each vial for 30 ± 5 s at high speed to recover the inoculum; ensure that the carrier is vortexing along with the liquid in the vial.

10.5.7 Visually examine each carrier (that is, look at the carrier through the bottom of the vial) and, in case of incomplete inoculum removal, perform further vortexing (for example, 30 ± 5 s) to remove inoculum. Do not remove the carrier from the vial.

10.6 *Dilution and Recovery:*

10.6.1 Vortex-mix the vial (10^0 dilution) for approximately 5 s and prepare serial ten-fold dilutions in PBS-T as necessary to achieve countable colonies in the target range of ~~20~~20 CFU to 200 CFU on the filters. Initiate dilutions within 30 min of neutralization.

10.6.2 For treated carriers, filter the entire contents of the vial (10^0 dilution) through a 0.2 µm PES membrane filter; the entire contents of other dilutions may be filtered as necessary. Initiate filtration within 30 min of preparing the dilutions.

10.6.3 Prior to filtration, pre-wet each membrane filter with approximately 10 mL PBS; apply vacuum to filter the contents. Leave the vacuum on for the duration of the filtration process.

10.6.3.1 To filter the contents of the vial, vortex-mix contents ~~(5~~(5 s to 10 s) and pour the contents into a filter unit. Rinse the vial with approximately 20 mL of PBS, vortex-mix for approximately 5 s and pour the entire contents of the vial into the same filter unit. Rinse the inside surface of each filter unit with an additional approximately 20 mL PBS.