Designation: E2839 – 21

Standard Practice for Production and Storage of Spores of *C. difficile* for Use in Efficacy Evaluation of Antimicrobial Agents[1]

This standard is issued under the fixed designation E2839; 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*

1.1 This practice specifies the procedures for producing and storing standardized suspensions of *Clostridioides difficile* spores for the evaluation of the sporicidal activity of antimicrobial formulations using the Quantitative Method for Testing Antimicrobial Agents against Spores of *C. difficile* on Hard, Non-porous Surfaces or other procedures.

1.2 This practice may involve hazardous materials, chemicals, and microorganisms and should be performed only by persons with formal training in microbiology.

1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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

1.5 *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:*[2]

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

E3218 Test Method for Quantitative Method for Testing Antimicrobial Agents against Spores of *C. difficile* on Hard, Nonporous Surfaces

3. Terminology

3.1 For definition of terms used in this practice refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *density gradient medium, adj/n*—HistoDenz (trademarked)[3] is a non-ionic gradient medium used here to separate spores from vegetative cells and cell fragments on the basis of density.

3.2.2 *pre-reduced medium, n*—an agar or broth manufactured in an oxygen-free environment and packaged in air-tight sealed pouches or bags.

3.2.3 *purified spores, n*—level of quality based on when spore concentration reaches ≥95 %, as vegetative cells and cell fragments are separated by the density gradient medium.

3.3 *Acronyms:*

CDC = Centers for Disease Control and Prevention
CFU = colony-forming unit
VFS = vegetative frozen stock.

4. Summary of Practice

4.1 This practice provides detailed instructions for the culture, maintenance, sporulation, and storage of spores of *C. difficile*. Spores are harvested from an agar medium following incubation in an anaerobic environment for 10 days. Upon harvesting, spores are washed several times with phosphate-buffered saline with polysorbate 80, exposed to heat to inactivate any vegetative cells, and purified using a density gradient medium. Purified spores are enumerated and assessed for quality using a carrier-based test employing two concentrations of sodium hypochlorite (NaOCl). Spores of *C. difficile* are stored for up to 90 days at -80 °C ± 5 °C.

5. Significance and Use

5.1 This practice describes a procedure for preparing and storing a suspension of *C. difficile* spores that meets the

[1] This practice 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] 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.

[3] The sole source of supply of HistoDenz (trademark) (Cat. No. D2158) known to the committee at this time is Sigma-Aldrich, St. Louis, MO. 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.

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

following acceptance criteria: *(1)* spore titer of approximately 5.0×10^8 spores/mL, *(2)* spore purity of ≥95 %, and *(3)* a mean $\log_{10}$ reduction (LR) value >5.0 for 3 carriers exposed to 5000 ppm and a mean LR of <3.0 for 3 carriers exposed to 1500 ppm sodium hypochlorite. These acceptance criteria are necessary in order to use the spore suspension to evaluate the performance of antimicrobial formulations using Test Method E3218.

6. Apparatus

6.1 *Calibrated micropipettes (for example, 200 µL and 1000 µL)*—1,000 µL pipettes with corresponding tips for transferring reagents and spores. 200 µL pipettes with corresponding tips for deposition of test substance on carrier.

6.2 *Sterile centrifuge tubes*—Polypropylene, 15 mL and 50 mL graduated plastic centrifuge tubes with conical bottoms.

6.3 *Microcentrifuge tubes*—Siliconized, sterile 1.5 mL low-retention microcentrifuge tubes. Use for dilutions and processing of spores during purification.

6.4 *Centrifuge with swinging-bucket rotor*—To allow sedimentation of spores for washing and/or concentration.

6.5 *Microcentrifuge*—To allow sedimentation of spores for washing and/or concentration.

6.6 *Inoculating loop*—10 µL loop to streak plates.

6.7 *Vortex mixer.*

6.8 *Polyethersulfone membrane filter (PES)*—For recovery of test spores, 47 mm diameter with 0.2 µm pore size. Use any filtration apparatus including filtration units (reusable or disposable).

6.9 *Anaerobic chamber*—Supported by a gas mixture consisting of 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. Alternatively, an activated anaerobic jar can be used according to manufacturer's instructions for ensuring an anaerobic environment.

6.10 *Incubator*—Use an incubator at 36 ± 1 °C inside an anaerobic chamber to support the growth of the organism. Alternatively, place anaerobic jars inside an aerobic incubator at 36 ± 1 °C.

6.11 *Microscope with 10× eyepiece and 100× (oil) objective with phase contrast option*—To evaluate purity of spore suspension.

6.12 *Timer*—Any timer that can display time in seconds.

6.13 *Cell scraper/spreader*—To aid in the removal of spores from agar plates.

6.14 *Laboratory film (or sealable bag)*—To seal inoculated plates during extended incubation (>48 h).

6.15 *Refrigerator (2-8 °C)*—For short term storage of spore suspension during the purification process.

6.16 *Freezer (-80 ± 5 °C)*—For long term storage of stock cultures and spore suspensions.

6.17 *Carriers*—Flat disks (1 cm in diameter) made of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side. See Test Method E3218 for carrier specifications.

6.18 *Calibrated 10 µL positive displacement pipette with corresponding 10 µL tips*—For carrier inoculation.

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

6.20 *Sterile forceps*—To handle membrane filters and to pick up the carriers for placement in vials.

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

6.22 *Sterile vials with lids (plastic or comparable)*—for holding inoculated carriers to be exposed to the test chemical and for accommodating the neutralizers. Flat-bottomed and wide-mouthed to accommodate addition and removal of the carriers. Use vials at least 25 mm in neck diameter and capable of holding at least 20 mL of liquid.

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

6.24 *Vacuum source*—In-house line or suitable vacuum pump (0.068 to 0.085 MPa) for drying carriers and for filtering.

6.25 *Digital titrator kit*—To measure total chlorine. Alternate titration methods may be used.

7. Media and Reagents

7.1 *Culture Media:*

7.1.1 *Reinforced clostridial medium (RCM)*—For use in rehydrating lyophilized/frozen vegetative culture of *C. difficile*. Prepare RCM according to manufacturer's instructions, and pre-reduce in an anaerobic environment for 24 h ± 2 h prior to use.

7.1.2 *RCM plus 15 % glycerol (Cryoprotectant)*—For use as cryopreservation medium for vegetative frozen stock (VFS) cultures. Prepare RCM and add 15 % (v/v) glycerol, autoclave for 20 min at 121 °C; pre-reduce in an anaerobic environment for at least 24 h ± 2 h prior to use.

7.1.3 *CDC anaerobic 5 % sheep blood agar (CABA)*—Sporulation medium commercially available pre-reduced.[4]

7.1.4 *Recovery media for enumeration of viable spores*—Pre-reduced brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT).[4]

7.2 *Reagents*[5]:

7.2.1 *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; PBS pH is 7.0 ± 0.5.

7.2.2 *Phosphate-buffered saline containing 0.1 % Tween 80 (PBS-T)*—Diluting and washing reagent; PBS-T pH is 7.2 ± 0.2.

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[4] The sole source of supply of the CABA (Cat. No. AS-646) and 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.

[5] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.