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.

**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**

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following acceptance criteria: *(1)* spore titer of approximately $5.0 \times 10^8$ 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.

7.2.3 *PBS-T with 0.1 % (w/v) sodium thiosulfate*—Neutralizer for sodium hypochlorite-based test chemicals; PBS-T with sodium thiosulfate pH is 7.2 ± 0.2.

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

7.2.5 *Reagent-grade sodium hypochlorite (NaOCl) with total chlorine ≥4%*—Use to prepare 5000 ± 250 ppm and 1500 ± 150 ppm total chlorine solutions to qualify spores.

7.2.6 *Tween-80 (polysorbate 80)*—Use to prepare PBS-T.

7.2.7 *HistoDenz™*—Use as a density gradient medium for spore purification. Prepare a 50 % (w/v) solution in deionized water. Pass the solution through a sterile 0.45 µm filter to sterilize. Store at 2-5 °C.

7.2.8 *Soil load*—Use in assay to qualify spore suspension. Soil load is a mixture of the following stock solutions:

7.2.8.1 *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.

7.2.8.2 *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.

7.2.8.3 *Mucin*—Add 0.04 g mucin (from bovine submaxillary gland or equivalent) to 10 mL of PBS, mix thoroughly until dissolved, and pass through a 0.2 µm pore diameter membrane filter.

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

NOTE 1—Other volumes of the stock solutions may be prepared following the same ratio.

## 8. Test Organism

8.1 *Clostridioides difficile* (ATCC 43598), formerly known as *Clostridium difficile*, a toxigenic strain (*tcdA*-, *tcdB*+), obtained from a reputable vendor. The strain produces Toxin B only (presence of *tcdB* gene by PCR). The organism is a Gram-positive, strictly anaerobic, spore-forming bacterium that produces flat, gray, irregular colonies on the surface of CABA plates within 48 h at 36 °C ± 1 °C.

## 9. Hazards

9.1 The test organism (*C. difficile*, ATCC 43598) must be incubated under strict anaerobic conditions and in accordance with local biosafety practices or those recommended by the U.S. Centers for Disease Control and Prevention/National Institutes of Health (CDC/NIH) for organisms at Biosafety Level II (**1**). Processing of spores can be conducted in an aerobic environment (for example, inside a BSC); all incubation for growth, however, **must** be performed anaerobically.

## 10. Preparation of Frozen Stock Cultures of Test Organism (Vegetative Form)

10.1 *Preparation of Inoculum:*

10.1.1 *C. difficile received in lyophilized vegetative form:*

10.1.1.1 In an anaerobic environment, reconstitute contents of the lyophilized culture with 0.5 mL of sterile pre-reduced RCM according to manufacturer's instructions.

10.1.1.2 After rehydration, aseptically transfer the vial contents to a tube containing 4 mL ± 1 mL of pre-reduced RCM, and mix by gentle vortexing.

10.1.2 *C. difficile received as frozen vegetative culture:*

10.1.2.1 Thaw frozen culture at room temperature.

10.1.2.2 In an anaerobic environment, transfer the contents to a tube containing 4 mL ± 1 mL of sterile pre-reduced RCM and mix by gentle vortexing.

10.2 *Inoculation of CABA Plates for Vegetative Stock Culture:*

10.2.1 Spread plate 100 µL of the reconstituted culture in RCM on each of five CABA plates.

10.2.2 Streak one CABA plate for isolation to check for culture purity.

10.2.3 Invert plates and incubate anaerobically at 36 °C ± 1 °C for 48 h ± 4 h. Observe CABA plate for purity and characteristics of *C. difficile*; discard the CABA plates if not pure and reinitiate a new stock culture.

10.3 *Harvest of CABA Plates for Stock Culture:*

10.3.1 Following incubation (10.2.3), add approximately 2 mL of sterile and pre-reduced cryoprotectant (7.1.2) to each CABA plate.

10.3.2 Using a sterile cell scraper, gently scrape culture from the surface of one plate, aspirate with a pipette and transfer to a 15 mL conical tube. Repeat this process for the remaining plates.

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10.3.3 Pool the suspensions, mix thoroughly, and pipette approximately 0.4 mL ± 0.1 mL aliquots into cryovials; cap tightly.

10.3.4 Store the cryovials at -80 °C ± 5 °C. These vials contain the Vegetative Frozen Stock (VFS) Culture.

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10.4 *Evaluation of VFS:*

10.4.1 Within 2-7 days after freezing, thaw a VFS cryovial at room temperature, preferably under anaerobic conditions. If processing under aerobic conditions, perform steps 10.4.1 – 10.4.4 within 15 min.

10.4.2 Vortex suspension thoroughly for at least 30 s ± 5 s and serially dilute 0.1 mL out to $10^{-6}$ in PBS-T.

10.4.3 Spread-plate 100 µL from $10^{-5}$ and $10^{-6}$ dilution tubes in duplicate on BHIY-HT.

10.4.4 Invert plates and incubate anaerobically at 36 °C ± 1 °C for 48 ± 4 h. Record the number of CFU/plate and determine the CFU/mL. The titer should be $>1.0\times10^{8}$ CFU/mL. Discard the VFS and reinitiate if the titer is $<10^{8}$ CFU/mL. If the titer is appropriate, use the frozen VFS for a maximum of 18 months, then reinitiate using a new lyophilized culture.

NOTE 2—New VFS culture may be initiated one time using an existing, unexpired frozen stock culture.

## 11. Preparation of a Test Spore Suspension from VFS

11.1 *Inoculation of CABA Plates:*

11.1.1 Thaw a VFS cryovial at room temperature. Streak three CABA plates with the VFS, preferably under anaerobic conditions.

11.1.1.1 Initiate anaerobic incubation for two plates and aerobic incubation for one plate at 36 °C ± 1 °C for 48 h ± 4 h within 15 min of removing the VFS cryovial from storage.

11.1.1.2 Inspect plates incubated anaerobically for purity and colony characteristics typical of *C. difficile*. Do not use the culture if there is uncharacteristic growth on any plate, or if there is any growth on the plate incubated aerobically.

11.1.2 Using one of the two anaerobic plates, inoculate 10 mL of pre-reduced RCM with an isolated colony and mix well by vortexing. Incubate anaerobically at 36 °C ± 1 °C for 24 h ± 2 h.

11.1.3 Vortex the RCM broth culture and use 100 µL to inoculate each of ten CABA plates. Spread the inoculum evenly over the plate using a sterile disposable spreader to create a lawn.

11.1.4 Seal inoculated plates with laboratory film to prevent dehydration during anaerobic incubation in an anaerobic chamber. Invert plates and incubate anaerobically for 10 days at 36 °C ± 1 °C and approximately 70 % relative humidity. Maintenance of relative humidity is not required if an anaerobic jar is used.

NOTE 3—Inspect one plate within 72 h of incubation to verify the presence of a lawn (confluent growth on the plate). If growth is confluent, reseal the plate and continue incubation.

11.1.5 Following the 10 day incubation period, transfer the CABA plates to a biological safety cabinet. Prepare wet-mount samples of *C. difficile* from a CABA plate and inspect under phase-contrast microscopy. Spores appear bright and ovular, while vegetative cells appear dark and rod-shaped. Degree of conversion of vegetative cells to spores should be approximately 90 % (see Fig. 1); if below approximately 90 %, discard the inoculated plates and initiate a new culture (11.1).

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11.2 *Harvesting CABA Plates:*

11.2.1 Harvest growth from each plate by adding 5 mL of PBS-T to each plate. Gently scrape the surface of the plate with a cell scraper or spreader to dislodge the spores. Do not break the surface of the agar.

11.2.2 Using a 10 mL sterile serological pipette, aspirate as much of the suspension as possible from each plate, pool it in a sterile 50 mL plastic conical tube, and mix well by thorough vortexing. Divide the suspension evenly into two 50 mL conical tubes. Mix well by thorough vortexing.

11.3 *Washing the Spore Suspension by Centrifugation:*

11.3.1 Centrifuge tubes from 11.2.2 at 4500×g for 15 min.

11.3.2 Discard the supernatant and resuspend the pellets with 30 mL of PBS-T per tube. Cap the tubes tightly and disaggregate the pellets by thorough vortexing. This step is the first wash. Centrifuge tubes at 4500×g for 15 min.

11.3.3 Discard the supernatant and resuspend the pellets with 30 mL of PBS-T. Cap the tubes tightly and disaggregate the pellets by thorough vortexing. This step is the second wash. Centrifuge tubes at 4500×g for 15 min. After the second wash, discard the supernatant and resuspend the pellets of one of the 50 mL conical tubes with 30 mL of PBS-T. Mix well by vortexing. Add the contents of the first tube to the second 50 mL conical tube. Mix well by thorough vortexing. This step is the third wash. Centrifuge tube at 4500×g for 15 min.

11.3.4 After the third wash, discard the supernatant and resuspend the pellet in 4 mL of PBS-T. Mix well by vortexing to disaggregate the pellet.

NOTE 4—For every 10 CABA plates inoculated, the resulting pellet is resuspended in 4 mL of PBS-T. Follow the ratio (4 mL per 10 plates) if additional plates are harvested.

11.4 *Heat Treatment:*

11.4.1 Heat the spore suspension in a heat block for 10 min ± 1 min at 65 °C ± 2 °C. To ensure that the spore suspension has reached 65 °C ± 2 °C, place a thermometer in an identical tube containing the same volume of PBS-T alongside the spore suspension (ensure that the thermometer is centered in the PBS-T and that the top of this tube is sealed around the thermometer) and start the timer once the temperature of the PBS-T in the tube has reached 65 °C ± 2 °C.

11.4.2 Following the heat treatment, allow the suspension to cool to room temperature.

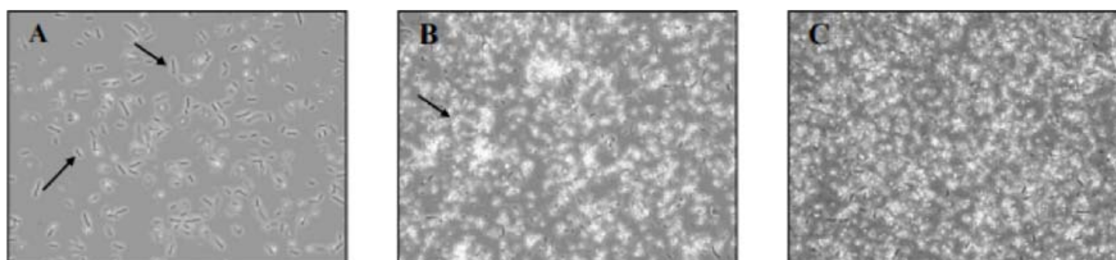
11.5 *Microscopic Evaluation of Spore Suspension*—Mix the spore suspension (11.4.2) by vortexing and prepare a wet-mount. Observe at least five fields using a phase-contrast microscope. The percent of spores to vegetative cells should be ≥90 %.

11.6 *Evaluate Titer of the Spore Suspension:*

11.6.1 Mix the spore suspension vigorously by vortexing (30 s ± 5 s) prior to taking an aliquot of the spore suspension (for example, 10 µL). Dilute the spore suspension out to $10^{-7}$ in PBS-T.

11.6.2 Spread-plate 0.1 mL of the $10^{-6}$ and $10^{-7}$ dilutions on BHIY-HT in duplicate.

11.6.3 Once the inocula have dried, invert plates and incubate anaerobically at 36 °C ± 1 °C for 48 h ± 4 h. Record the




(A) Arrows indicate vegetative cells after 2 days of incubation. (B) Arrow indicates spores after 8 days of incubation. (C) Final harvested spore preparation after 10 days of incubation (approximately 90 % spores).

**FIG. 1 Sporulation of *C. difficile* (ATCC 43598) during incubation at 36 ± 1 °C using phase contrast microscopy (magnification 1000 ×).**