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Standard Test Method for Production of *Clostridium difficile* Spores 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.

INTRODUCTION

Sporulation in *Clostridium difficile* is not as rapid or as efficient as in other species and it is generally difficult to produce *C. difficile* spores of high titer in the laboratory (**1, 2**).[2] Although quantitative test methods are available for testing sporicidal products, a standardized method for generating spore suspensions of *C. difficile* of high titer (>8 $\log_{10}$/mL) and purity (≥95 % spores) is not available and would be necessary in order to conduct performance testing required for registration purposes (**3**). The spore suspensions resulting from practice of this test method are appropriate for use in accepted test methods for measuring the sporicidal efficacy of antimicrobial formulations (**4**).

1. Scope

1.1 This test method is for producing *C. difficile* spores to evaluate antimicrobial formulations for their sporicidal activity.

1.2 It is the responsibility of the investigator to determine whether Good Laboratory Practices (GLP) are required and to follow them when appropriate.

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

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

2. Terminology

2.1 *Definitions:*

2.1.1 *CFU, adj/n*—colony-forming units; the number of spores or microorganisms that can form colonies (clusters of microorganisms visibly growing on the surface of a solid agar medium) in spread plates, as an indication of the total number of viable spores/microorganisms in a sample.

2.1.2 *QC, adj/n*—quality control (QC) is the application of procedures, products, or services to meet a laboratory's specified standards of quality.

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

2.1.4 *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.

2.1.5 *purified spores, adj/n*—when spore concentration reaches ≥95 % as vegetative cells and cell fragments are separated by the density gradient medium.

2.1.6 *toxigenic strain, adj/n*—possesses either toxin A gene (*tcdA*+) or toxin B gene (*tcdB*+) or both.

3. Summary of Test Method

3.1 This test method provides detailed instructions for the culture, maintenance and sporulation of *C. difficile* on a specific agar medium incubated in an anaerobic environment for 7 to 10 days. Monitoring is performed by phase-contrast microscopy to ensure sporulation is underway and to determine when the spore concentration reaches ≥90 %, the optimal time of harvest. Upon harvesting, spores are washed several times with saline-Tween 80, treated with heat to inactivate any remaining viable vegetative cells, and purified using a density gradient medium to remove inactivated vegetative cells and cell fragments, with a target spore-purity of ≥95 %. Purified spores are

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[2] The boldface numbers in parentheses refer to a list of references at the end of this standard.

[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.

enumerated on specific agar-based recovery medium for titer determination and assessed for quality using a quantitative acid-resistance test.

4. Significance and Use

4.1 This test method describes a procedure for preparing a spore suspension of *C. difficile* strain ATCC 43598 that meets specific criteria necessary for efficacy testing of antimicrobials designed to eliminate *C. difficile* contamination from environmental surfaces. The acceptability criteria for the spore suspension are: (*1*) a viability titer of >8 $\log_{10}$/mL, (*2*) purity of ≥95 %, and (*3*) that spores be resistant to 10 min of exposure to 2.5 M HCl.

5. Apparatus

5.1 *Biosafety cabinet (BSC, Type B2, Class II)*—Recommended for maintaining an aseptic work environment.

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

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

5.4 *Micropipette*—Calibrated.

5.5 *Positive displacement pipette*—To inoculate steel carriers with spores.

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

5.7 *Test tubes*—Reusable or disposable 20 × 150 mm for cultures/subcultures.

5.8 *Inoculating loop*—10 µL transfer loop.

5.9 *Anaerobic chamber*—Supported by a gas mixture consisting of 10 % hydrogen, 5 % CO_2, and 85 % N_2. Alternatively, an activated anaerobic jar can be used according to manufacturer's instructions for ensuring an anaerobic environment.

5.10 *Anaerobic incubator*—Use an incubator at 36 ± 1°C placed inside the anaerobic chamber to support the growth of the organism. Alternatively, use an activated anaerobic jar containing inoculated plates that is placed inside an aerobic incubator at 36 ± 1°C. Plates must be incubated in an anaerobic environment at 36 ± 1°C for growth to occur.

5.11 *Microscope with 10× eyepiece and 40× and 100× (oil) objectives with phase contrast option.*

5.12 *Vortex mixer.*

5.13 *Serological pipettes*—Sterile single-use pipettes of 10.0, 5.0, 1.0 mL capacity.

5.14 *Cell Scraper*—To gently scrape plates to remove spores for harvesting.

5.15 *Plate spreader*—To spread inocula on agar to create a uniform lawn.

5.16 *Microcentrifuge tubes*—Sterile 1.5-mL low-retention (siliconized) microcentrifuge tubes.

5.17 *Cryovials*—Sterile 2.0 mL cryovials.

5.18 *Parafilm.*

6. Media and Reagents

6.1 *Culture Media*:

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

6.1.2 *RCM plus 15 % glycerol (Cryoprotectant)*—For use as maintenance and cryopreservation medium for vegetative frozen stock (VFS) cultures. Prepare RCM and add 15 % glycerol, autoclave for 20 min at 121°C, and pre-reduce (6.1.1).

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

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

6.2 *Phosphate-buffered saline (PBS)*—Prepare 10× stock solution of PBS by dissolving 492 g PBS powder in 5 L of deionized water. Dilute 1:10 (1 part 10× solution plus 9 parts deionized water) to obtain 1× solution, distribute into bottles and autoclave for 20 min at 121°C.

6.3 *Phosphate-buffered saline (PBS) containing 0.1 % Tween 80 (ST80)*—Washing reagent; add 2.0 mL of polysorbate 80 (Tween 80, or equivalent) to 2.0 L of PBS (1×) solution in a 2 L volumetric flask and bring solution to volume with PBS. Distribute into bottles and autoclave for 20 min at 121ºC.

6.4 *Water*—Sterile deionized water (**5**).

6.5 *Hydrochloric acid*—Prepare 2.5 M HCl from 5 M HCl.

6.6 *HistoDenz*—Prepare a 50 % (w/v) solution in deionized water. This is a density gradient medium. Pass the solution through a sterile 0.45 µm filter.

7. Test Organism

7.1 *Clostridium difficile* (ATCC 43598), a toxigenic strain (*tcdA*-, *tcdB*+), can be 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, and irregular colonies on the surface of CABA medium within 48 h at 36 ±1°C.

8. Hazards

8.1 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 (**6**). Processing of spores can be conducted in an aerobic environment (for example, inside a BSC); all incubation for growth, however, **must** be performed anaerobically.

8.2 Use suitable personal protective equipment (PPE) and other appropriate safety devices when handling hydrochloric acid and other hazardous chemicals. Consult relevant Material Safety Data Sheets (MSDS) in advance for specific details on safe manipulation of such chemicals and corrective action in case of spills or exposure.

[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.

9. Preparation of Frozen Stock Cultures of Test Organism

9.1 *Preparation of Inoculum*:

9.1.1 *Clostridium difficile received in lyophilized vegetative form*:

9.1.1.1 Reconstitute contents of the lyophilized culture with 0.5 mL of sterile pre-reduced RCM in an anaerobic environment according to manufacturer's instructions.

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

9.1.2 *Clostridium difficile received as frozen vegetative culture*:

9.1.2.1 Thaw frozen culture at room temperature.

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

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

9.2.1 Inoculate by spread-plating each of five CABA plates (100-cm diameter) with 100 µL of the reconstituted/diluted culture of *C. difficile*.

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

9.2.3 Invert plates and incubate anaerobically at 36 ± 1°C for 48 ± 4 h.

9.3 *Harvest of CABA Plates for Stock Culture*:

9.3.1 Following incubation (9.2.3), add approximately 2 mL of sterile and pre-reduced cryoprotectant (6.1.2) to each CABA plate.

9.3.2 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.

9.3.3 Pool the cryoprotectant suspensions, mix thoroughly, and pipette 1 to 1.5 mL aliquots into cryovials; cap tightly.

9.3.4 Store the cryovials at ≤ –70°C. These tubes are the Frozen Stock Culture (FSC).

9.4 *Evaluation of Viable Titer of FSC*:

9.4.1 Approximately 7 ± 1 days after freezing, thaw a stock culture cryovial at room temperature inside an anaerobic chamber.

9.4.2 Vortex suspension thoroughly, and dilute 1 mL in a 1:10 series out to 10^{-6} in ST80 (6.3).

9.4.3 Spread-plate 100 µL of diluted suspension on BHIY-HT in duplicate.

9.4.4 Invert plates and incubate anaerobically at 36 ± 1°C for 48 ± 4 h. Record the number of CFU/plate to determine the viable titer/mL, which should be >8 $\log_{10}$/mL to ensure that FSC contains a sufficiently high titer to withstand long-term storage at ≤ –70°C.

10. Preparation of a Test Spore Suspension from FSC

10.1 *Inoculation of CABA Plates*:

10.1.1 As a part of QC, streak three CABA plates with a frozen stock culture of test organism. Incubate two plates anaerobically, and the third one aerobically at 36 ± 1°C for 48 ± 4 s. Do not use the culture if there is any growth on the plate incubated aerobically. Inspect plates incubated anaerobically for purity and colony characteristics typical of *C. difficile*.

10.1.2 Inoculate 10 mL of pre-reduced RCM with an isolated colony from a CABA plate and mix well by vortexing. Incubate anaerobically at 36 ± 1°C for 24 ± 2 h.

10.1.3 After incubation, inoculate each of a minimum of ten CABA plates with 100 µL of the RCM broth culture. Spread the inoculum evenly using a disposable sterile spreader to create a lawn.

10.1.4 Seal culture plates with Parafilm, or equivalent, to prevent dehydration during the extended anaerobic incubation. Invert plates and incubate anaerobically for 7 to 10 days at 36 ± 1°C and ≥70 % relative humidity. Maintenance of relative humidity is not required if an anaerobic jar is used.

10.1.5 Open one or two plates after about 24 h of incubation to inspect for confluent growth. Do not continue with the incubation if growth is not confluent. Wet-mount samples of *C. difficile* from the plates periodically during the first 2 to 6 days of incubation, and daily on days 7 to 10, for inspection under phase-contrast microscopy. Note degree of conversion of vegetative cells to spores and estimate the approximate ratio of spores to vegetative cells to determine the optimal time for harvesting. Under phase-contrast, spores appear bright and ovular, while vegetative cells appear dark and rod-shaped.

10.2 *Harvesting CABA Plates Inside a BSC (that is, aerobic environment)*:

10.2.1 When the percent of spores reaches ≥90 %, discontinue incubation in anaerobic environment and remove the CABA plates into a BSC. Harvest growth from each plate by adding approximately 5 mL of ST80 to each plate, and gently scrape the surface of the plate with a cell scraper to dislodge the spores. Do not break the surface of the agar, and avoid collecting agar fragments, insofar as possible.

10.2.2 Using a 10 mL sterile serological pipette, aspirate as much of the microbial suspension as possible from each plate, and pool it in sterile 50-mL plastic conical tubes. Cap the tubes tightly for centrifugation. For proper balancing, there must be at least two 50-mL plastic tubes of the same size with the same volume, and pairs of tubes must be positioned in buckets diametrically opposite one another.

10.3 *Washing the Spore Suspension by Centrifugation*:

10.3.1 Centrifuge tubes at 4500 × g for 15 min.

10.3.2 Discard the supernatant and resuspend the pellet in 20 to 30 mL of ST80. Cap the tubes tightly and disaggregate the pellet by vortex-mixing. This step is the first wash.

10.3.3 Repeat the washing step two more times. Note that resuspended contents collected from two or more tubes can be combined in one tube only after pellets have been disaggregated. Mix by vortexing.

10.3.4 After the third wash, discard the supernatant and resuspend the pellet in about 4 mL of ST80. Mix well by vortexing to disaggregate the pellet. This is the working spore suspension.

10.4 *Heat Treatment*:

10.4.1 Heat the working spore suspension in a water-bath or a heat block for l0 ± 1 min at approximately 65 ± 2°C. To ensure that the spore suspension has reached 65 ± 2°C prior to starting the timer, place a thermometer in an identical tube

containing the same volume of deionized water alongside the spore suspension and start the timer once the temperature of the water has reached 65 ± 2°C.

10.4.2 On elapse of the 10 ± 1 min exposure, allow the suspension to cool to room temperature.

10.5 *Microscopic Evaluation of Working Spore Suspension (predominantly spores, along with dead vegetative cells and cell fragments)*—Prepare a wet-mount of the well-vortexed, heat-treated working spore suspension (10.4.2) and observe at least five fields using a phase-contrast microscope. The spore concentration should be ≥90 %.

10.6 *Evaluate Titer of the Spore Suspension*:

10.6.1 Perform serial 10-fold dilutions of the spore suspension out to 10^{-6} in ST80.

10.6.2 Spread-plate 0.1 mL of the appropriate dilutions on BHIY-HT in duplicate.

10.6.3 Once the inocula have dried, invert plates and incubate anaerobically at 36 ± 1°C for 48 ± 4 h. Record the numbers of CFU. The titer should be $>10^8$ viable spores/mL.

11. Spore Purification

11.1 Make a 50 % (w/v) solution of HistoDenz in sterile deionized water (6.6).

11.2 Pipet 5 mL of sterile 50 % (w/v) HistoDenz into each of four sterile 15-mL plastic conical tubes.

11.3 Layer 1 mL of spore suspension (10.4.2) on top of 5 mL of 50 % (w/v) HistoDenz in each of four 15-mL plastic conical tubes.

11.4 Centrifuge tubes at 4500 × g for 10 min using a swinging bucket rotor (see Note 1). Four layers will be formed in the HistoDenz solution, with spores aggregated in the bottom layer, mostly pelleted. Avoid disturbing pellet and the cloudy layer about 3 to 4 mm above the pellet.

NOTE 1—Use of a swinging bucket rotor is essential for proper layer removal and spore retention.

11.5 Carefully remove with a 1 mL pipet the top three layers—an upper clear layer, a dense second layer, and a clear third layer—and discard, leaving the pellet and 3 to 4 mm cloudy layer above the pellet undisturbed.

11.6 Use a pipette to resuspend the pellet, mix by vortexing and transfer approximately 1 mL aliquots to siliconized microcentrifuge tubes until the entire volume has been transferred.

11.7 Centrifuge the microcentrifuge tubes at 16 000 × g for 5 min.

11.8 Discard the supernatant and resuspend the pellet in 1 to 1.5 mL of cold (2 to 5°C) ST80. Cap the tubes and mix by vortexing to thoroughly disaggregate the pellet.

11.9 Centrifuge the microcentrifuge tubes at 16 000 × g for 2 min. Discard the supernatant and resuspend the pellet in 1 to 1.5 mL of cold (2 to 5°C) ST80. Cap the tubes and mix by vortexing to thoroughly disaggregate the pellet. This step is the first wash.

11.10 Repeat 11.9 procedures two additional times, for a total of three washes. Discard the supernatant and resuspend the pellet in each microcentrifuge tube in 0.5 mL of sterile ST80. This is the final working suspension (purified).

NOTE 2—Resuspended contents of microcentrifuge tubes can be combined, as necessary.

11.11 Determine spore purity using procedures stated in 10.5, and calculate purity of the spore suspension using the formula presented in 13.1.

11.12 Perform procedures specified in 10.6, and calculate the titer of the purified spore suspension using the formula presented in 13.2.

12. Quantitative Acid Resistance Test

12.1 *HCl Resistance*:

12.1.1 Place 990 µL of 2.5 M HCl into one 1.5 mL low-retention (siliconized) microcentrifuge tube; for the control, place 990 µL of ST80 into one 1.5 mL low-retention (siliconized) microcentrifuge tube.

12.1.2 Using a positive-displacement pipette, transfer 10 µL of purified spore suspension (spore titer of >8 $\log_{10}$/mL) into each microcentrifuge tube to result in a suspension containing $>10^6$ spores/mL. Vortex each tube.

12.1.3 Incubate the acid/spore suspensions and the control tubes for 10 min at room temperature.

12.1.4 At the end of each incubation period, transfer 0.1 mL from the acid/spore tube and the control tube to tubes containing 900 µL of ST80 to dilute/neutralize the acid.

12.1.5 Serially dilute the neutralized suspensions out to 10^{-6} in ST80 and spread-plate 0.1 mL aliquots from appropriate dilutions, in duplicate, on BHIY-HT. Invert plates and incubate for 48 ± 4 h at 36 ± 1°C under anaerobic conditions.

12.1.6 The spores are considered acid-resistant if their $\log_{10}$ viability is between 0 to 2 following 10 min of exposure, as compared with the control.

13. Calculation

13.1 Determine spore suspension purity:

$$\textit{Spore Purity} = 100\ \% \times \frac{A}{A+B} \tag{1}$$

where:

A = mean spore count, and
B = mean vegetative cell count.

13.2 Determine titer of viable purified spores as CFU/mL:

$$\textit{Purified Spores as CFU/mL} = \frac{A \times B}{C} \tag{2}$$

where:

A = mean colony count at dilution plated,
B = reciprocal of dilution used, and
C = volume plated.

13.3 Determine the $\log_{10}$ reduction following HCl treatment:

$$\log_{10} Reduction\ (LR) = LC - LH \quad (3)$$

where:
LC = $\log_{10}$ of viable spores after control treatment, and
LH = $\log_{10}$ of viable spores after HCl treatment.

14. Precision and Bias

14.1 A precision and bias statement cannot be made at this time.

15. Keywords

15.1 acid resistance; *Clostridium difficile*; density gradient medium; spore production; spore purity; sporicidal efficacy testing; vegetative cells

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[5] 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.