Designation: ~~E2895 – 13~~ E2895 – 19

Standard ~~Test Method~~ Practice for Producing High Titers of Viable and Semi-Purified Spores of Clostridium difficile using a Liquid Medium[1]

This standard is issued under the fixed designation E2895; 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

Clostridium difficile (*C. difficile*), an anaerobic spore-former, can cause acute and potentially fatal gastroenteritis in healthcare and other settings (**1**).[2] The frequent episodes of diarrhea can contaminate the indoor environment widely with persistent (**2**) and microbicide-resistant (**3**) spores. Disinfectants wishing to claim activity against *C. difficile* now require carrier testing using spores of high purity (>90%) that show a >6 $\log_{10}$ reduction in spore viability (**4**). While the use of a semi-solid medium for *C. difficile* spore production has been reported (**5**) (Test Method E2839), this standard describes a liquid medium and an enzyme-based semipurification ~~process for the purpose.~~ process. Appendix X1 describes an alternative to enzyme purification.

~~1. Scope~~

~~1.1 This test method describes the production and semipurification of *C. difficile* spores (also called endospores) primarily for use in testing the sporicidal activities of environmental surface disinfectants (Test Methods E2111 and E2197); such spores can also be used to study their structure, chemistry and germination.~~

~~1.2 While the test method described is based on the use of 500-mL volumes of the liquid culture medium in an anaerobic incubator, anaerobic jars with smaller volumes of the same medium can also be used.~~

~~1.3 It is the responsibility of the investigator to determine whether Good Laboratory Practice (GLP) regulations are required and to follow them when appropriate (40 CFR, Part 160 for EPA submissions and 21 CFR; Part 58 for FDA submissions).~~

~~1.4 **Warning—**This standard may involve hazardous materials, chemicals, and microorganisms and should be performed only by persons with formal training in microbiology.~~

~~1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.~~

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

~~2. Referenced Documents~~

~~2.1 *ASTM Standards:*[3]~~

~~D1129 Terminology Relating to Water~~

~~E2111 Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal, and Sporicidal Potencies of Liquid Chemicals~~

~~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~~

[1] This ~~test method~~ 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] The boldface numbers in parentheses refer to a 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.

E2839 Test Method for Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents

2.2 *Federal Standards:*[4]

21 CFR, Part 58 Good Laboratory Practice for Nonclinical Laboratory Studies

40 CFR, Part 160 Good Laboratory Practice Standards

3. Terminology

3.1 *Definitions*—For definitions of general terms used in this test method, refer to Terminologies D1129 and E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *anaerobe, n*—an organism that cannot grow or proliferate in the presence of free oxygen.

3.2.2 *enzymatic treatment, n*—use of one or more enzymes to digest remnants of cells in spore suspensions.

3.2.3 *germination, n*—when a spore re-emerges into its vegetative form for active growth and replication.

3.2.4 *spore, n*—phase of dormancy in the reproductive cycle of certain types of microorganisms where individual cells become condensed in a relatively impermeable coat, enabling prolonged survival and greater resistance to deleterious environmental factors.

3.2.4.1 *Discussion*—

A spore can germinate into a vegetative cell under favorable conditions to reinitiate the cycle of replication.

4. Summary of Test Method

4.1 This standard relates to a new liquid culture medium together with a semipurification process (**2**) designed to produce high titers (>10^9 CFU/mL) of viable spores of *C. difficile* with a purity of >90%. In this method, the semipurification of the spores is achieved by enzyme treatment. A surcose density gradient method (Appendix X1) is also described as an alternative to enzyme treatment.

5. Significance and Use

5.1 The quantity and quality of the spores produced by this method meet the current requirements of the U.S. Environmental Protection Agency (EPA) to assess environmental surface disinfectants for label claims of sporicidal activity (**4**). The method is applicable to standard as well as clinically isolated toxigenic and non-toxigenic strains of *C. difficile*.

6. Reagents, Materials, and Equipment[5]

6.1 *Chemicals and Reagents:*

6.1.1 *Columbia Broth (CB) Powder*.

6.1.2 *Special Peptone Mix (SPM)*.

6.1.3 *Brain-Heart Infusion (BHI) Broth Powder*.

6.1.4 *Yeast Extract Powder*.

6.1.5 *Bacteriological Agar*.

6.1.6 *Lysozyme* (egg white).

6.1.7 *Trypsin (porcine pancreas)*—lyophilized.

6.1.8 *Disodium Hydrogen Phosphate* (Na_2HPO_4).

6.1.9 *Sodium Dihydrogen Phosphate* ($NaH_2PO_4·H_2O$).

6.1.10 *Potassium Hydroxide* (KOH).

6.1.11 *Sodium Hydroxide* (NaOH).

6.1.12 *Hydrochloric Acid* (HCl).

6.1.13 *Potassium Dihydrogen Phosphate* (KH_2PO_4).

6.1.14 *Potassium Carbonate* (K_2CO_3).

6.1.15 *Calcium Chloride* ($CaCl_2·2H_2O$).

6.1.16 *Ammonium Sulfate* $(NH_4)_2SO_4$).

6.1.17 *Magnesium Sulfate* ($MgSO_4$).

6.1.18 *Taurocholic Acid Sodium Salt Hydrate*.

6.1.19 *L-Cysteine*.

6.1.20 *Phosphate Buffered Saline* (PBS)—0.85% NaCl in 0.3 mM phosphate buffer (pH 7.2 ± 0.1).

[4] Available from Superintendent of Documents, U.S.S Government Printing Office, Washington DC, 20402. http://www.gpo.gov/

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual 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.

6.1.21 *PBS Tween*[6]80 (PBS-T)—0.85% NaCl and 0.1% (v/v) Tween 80 in 0.3 mM phosphate buffer (pH 7.2 ± 0.1).
6.1.22 *0.1 M Sodium Phosphate Buffer*—pH 7.0 ± 0.2.
6.1.23 *Deionized Water*—or water of equivalent purity.
6.1.24 *Tryptic Soy Agar Plates (TSA)*—to test the final spore suspension for contamination.

6.2 *Equipment:*
6.2.1 *Anaerobic Incubator*—with gas supply: 5% CO_2, 10% H_2, 85% N_2, capable of maintaining 36 ± 1ºC.

NOTE 1—Anaerobic jars with suitable gas packs may also be used.

6.2.2 *Analytical Balance.*
6.2.3 *Disposable or Reusable Membrane Filter Holders,* for 47-mm diameter membrane filters.
6.2.4 *Bench-top Centrifuge.*
6.2.5 *Centrifuge with Fixed-angle and Swinging-bucket Rotors,* to process spore suspensions.
6.2.6 *Forceps,* straight or curved with smooth tips to handle membrane filters.
6.2.7 *Freezers,* one at -20 ± 2°C to store media and additives, and another at -70°C or lower to store stocks of microorganisms.
6.2.8 *Hot Plate with Stirrer*—to prepare culture media and reagents.
6.2.9 *Laminar-flow Biological Safety Cabinet—Class II, Type A*—procedures for proper maintenance and use of such cabinets are given in the reference section (**6**).
6.2.10 *Light Microscope,* with an oil-immersion objective to observe and count spores and vegetative cells in stained slides to assess the level of spore purity.
6.2.11 *Standard Glass Microscope Slides and Coverslips*
6.2.12 *pH Meter,* with electrodes and standard solutions.
6.2.13 *Pipettors,* in the following sizes: 1-20 µL, 20-100 µL, and 200-1000 µL.
6.2.14 *Positive-displacement Pipette,* 2-20 µL.
6.2.15 *Refrigerator,* capable of maintaining 4 ± 1°C for storage of culture media, culture plates, and reagents.
6.2.16 *Sterile Serological Pipettes,* 1, 5, 10, and 25-mL capacity.
6.2.17 *Sonicator Bath,* ~ 40 KHz; recommended to break down spore clusters.
6.2.18 *Sterilizer,* any steam sterilizer suitable for processing culture media, reagents, and labware.
6.2.19 *Vacuum Source,* a vacuum pump, access to an in-house vacuum line, or a water faucet vacuum apparatus, to pull samples through membrane filters.
6.2.20 *Vortex Mixers,* one for use outside and another to remain inside the anaerobic chamber.
6.2.21 *Waterbath,* for heat-inactivation of vegetative cells in spore suspensions at 69 ± 1°C.

6.3 *Labware*—(unless otherwise indicated, all labware must be sterile):
6.3.1 *Plastic Transfer Pipettes,* Pasteur pipettes.
6.3.2 *Polypropylene Tubes,* 15 and 50-mL screw-cap.
6.3.3 *Glass Culture Flasks,* 1 L.
6.3.4 *Pipette Tips,* 20, 200, and 1000 µL.
6.3.5 *Polycarbonate Centrifuge Tubes,* 40 and 250 mL.
6.3.6 *Cryovials,* 2 mL.
6.3.7 *Membrane Filters,* 47-mm diameter; 0.22-µm pore diameter.
6.3.8 *Syringe-driven Filter Units,* 20-mm diameter; 0.22-µm pore diameter; low protein-binding.
6.3.9 *Petri Dishes,* 100 mm in diameter, for spore recovery medium.

6.4 *C. difficile* Strain:
6.4.1 While much of this method is based on work with ATCC 43598, the procedures described are suitable for other standard strains, and also clinical isolates of the organism.

7. Methods

7.1 *Preparation of Media:*
7.1.1 *Columbia Broth (CB):*
7.1.1.1 Prepare a 1× solution of CB following the manufacturer's instructions.
7.1.1.2 Sterilize by autoclaving and store refrigerated.
7.1.2 *Liquid Sporulation Medium:*
7.1.2.1 Prepare 1 L of the medium in a 2-L Erlenmeyer flask by adding the following in the order given:

[6] Trademark ICI Americas.

Deionized water	700 mL
SPM	10.0 g
KH2PO4	2.60 g
$(NH_4)_2SO_4$	0.60 g
$CaCl_2{\cdot}2H_2O$	0.08 g
Yeast extract powder	10.0 g
K_2CO_3	3.48 g
$MgSO_4$	0.12 g
Deionized water to 1 L	

7.1.2.2 The pH of the medium should be 7.9 ± 0.2 before autoclave sterilization; if needed, adjust with 1 M KOH.

7.1.2.3 Put 500 mL of the medium into two 1-L flasks and autoclave for 20 min at 121ºC.

7.1.2.4 Wait about 3 h for the temperature to drop to 50–60ºC.

7.1.2.5 Place the flasks in an anaerobic incubator (36 ± 1°C) for 18-24 h to prereduce the medium; during this period the pH of the medium should rise to 8.2 ± 0.3.

7.1.2.6 The liquid culture medium must be prepared the day before its use so that it is already prereduced on the day it is to be inoculated.

7.1.3 *Agar Medium for Spore Recovery (BHIYT-L):*

7.1.3.1 Add the following to 1 L of deionized water:

BHI	37.0 g
Yeast extract powder	5.0 g
L-Cysteine	1.0 g
Sodium taurocholate	1.0 g
Bacteriological agar	15 g

7.1.3.2 Boil the medium for 1 min to dissolve the ingredients and then autoclave it for 20 min at 121ºC; let the medium cool down to 60 ± 2°C.

7.1.3.3 Dissolve 200 000 units of lysozyme in 10 mL of deionized water; put the enzyme solution in a syringe-driven membrane filter and add it directly to the medium.

7.1.3.4 Pour the medium immediately into culture plates. Such plates can be stored refrigerated for no longer than six months. No prereduction of the medium in the plates is necessary when used for spore recovery.

NOTE 2—As shown in a three-laboratory collaborative (7), certain types of commercially available horse-blood containing recovery media may be used instead.

7.2 *Preparation of Reagents:*

7.2.1 *1M Sodium Phosphate Buffer (pH 7.0):*

7.2.1.1 Dissolve in 800 mL of deionized water: 8.1934 g of anhydrous Na_2HPO_4 and 5.8374 g of $NaH_2PO_4{\cdot}H_2O$ in a 2-L flask.

7.2.1.2 Adjust pH to 7.0 ± 0.2 with 1 M NaOH or 1 M HCl.

7.2.1.3 Add more deionized water to give a total volume of 1 L.

7.2.1.4 Autoclave for 20 min at 121°C.

7.2.2 *Enzyme Mixture:*

7.2.2.1 First determine the wet weight of the spore pellet (see 8.4). Then add 800 units of lysozyme and 250 units of trypsin per mg of pellet wet weight to 25 mL of 0.1 M phosphate buffer (pH 7.0).

7.2.2.2 Proceed as described in 8.4.

8. Procedure

8.1 *Inoculum Preparation:*

8.1.1 Take a loopful from the spore stock and streak it onto a BHIYT-L plate.

8.1.2 Incubate the plate anaerobically at 36 ± 1°C for 48 ± 4 h.

8.1.3 At the same time, keep in the anaerobic chamber for pre-reduction *(a)* one 15-mL plastic tube containing 5 mL of CB to prepare the pre-inoculum and *(b)* the same number of 50-mL conical plastic tubes, each with 20 mL of CB, as the number of culture flasks to be inoculated.

8.1.4 Pick an isolated colony from the inoculated BHIYT-L plate and suspend it in the tube containing 5 mL of prereduced CB; incubate the tube anaerobically at 36 ± 1°C for 24 to 36 h.

8.1.5 Inoculate 50 µL of the 24 to 36-h culture from 8.1.3 into each 50-mL conical tube containing 20 mL of prereduced CB; incubate anaerobically at 36 ± 1°C for 18 ± 2 h.

8.1.6 Prepare the required volume of liquid sporulation medium as described in 7.1.2.1.

8.2 *Culture Flask Inoculation and Incubation Time:*

8.2.1 Inside the anaerobic chamber, pour the entire inoculum from a 20-mL tube into a 500-mL culture flask with the liquid sporulation medium.

8.2.2 Incubate the flask(s) anaerobically in a stationary state for 5 days at 36 ± 1ºC.

8.3 *Harvesting:*

8.3.1 Divide the sporulated culture from each flask equally into two 250-mL centrifuge tubes and centrifuge the suspensions at 4 000xg for 10 min.

8.3.2 Discard the supernatant as biohazardous waste.

8.3.3 Resuspend the sediment from each 250-mL tube into 50 mL of deionized water and pool the resuspended material into one 250-mL centrifuge tube. Adjust the volume to 250 mL with deionized water and centrifuge for 10 min at 4000xg. Wash the pellet by centrifugation two more times with 250 mL of deionized water in each wash.

8.3.4 After the final wash, resuspend the pellet in 15 mL of PBS-T and transfer the suspension into a preweighed 40-mL centrifuge tube. Rinse the original centrifuge tube once with 10 mL PBS-T and add the wash to the spore suspension to make the final volume 35 mL.

8.3.5 Centrifuge the suspension at 4 000xg for 10 min, and wash the pellet twice with 35 mL of PBS-T. Discard all supernatants as biohazardous waste.

8.3.6 Weigh the tube with the pellet and refrigerate (4 ± 1°C), for no longer than 48 h, until the next step.

8.3.7 Use the wet weight of the pellet to determine the quantities of the enzymes needed in the solution for semipurification of the spore suspension.

8.4 *Semipurification of the Spore Suspension by Enzyme Treatment:*

8.4.1 Resuspend the pellet (1-700 mg of wet weight) in 10 mL of 0.1 M sodium phosphate buffer (pH 7.0 ± 0.2); if the wet weight of the pellet is higher than 700 mg, divide the suspension equally into two separate tubes and adjust the volume in each to 10 mL; mix well by vortexing.

8.4.2 Add 25 mL of a freshly prepared mixture of the enzymes as described above in 7.2.2, and mix gently, but do not vortex.

8.4.3 Sonicate the suspension at ~40 KHz in an ultrasonic waterbath for 5 min and then incubate it at 45 ± 1ºC for 6 h; repeating the sonication step after 2 ± 0.2 h, 4 ± 0.2 h and 6 ± 0.2 h of incubation to break up the clumps, and then store the suspension refrigerated overnight.

8.4.4 Centrifuge the suspension at 4000xg for 10 min, and wash the pellet three times with 10 mL of PBS-T in each wash.

8.4.5 Resuspend the pellet in 30 mL of PBS-T and heat it for 10 min in a waterbath at 69 ± 1ºC. Insert a thermometer in an identical tube with the same volume of water to determine when the temperature has reached the desired level and start counting the heating time at that point.

8.4.6 Immediately at the end of the heating time, submerge the tube in ice for 5 min.

8.4.7 Centrifuge the suspension at 4000xg for 10 min, resuspend the pellet in 2 mL PBS-T for each 500 mL culture volume used. Such spore suspensions can be stored refrigerated (4 ± 1ºC) and used over a period of no longer than six months.

9. Quality Control

9.1 *Testing for contamination with aerobic organisms:*

9.1.1 To ensure that the final spore suspension is free from aerobic organisms streak a loopful of the suspension onto two plates of TSA and incubate one at 30 ± 1°C and the other at 36 ± 1°C for 48 ± 4 h. The absence of any growth would indicate the freedom of the spore suspension from common heterotrophic aerobic bacterial contaminants.

10. Phase-Contrast Microscopy and Spore-Staining of Final Spore Suspensions

10.1 Prepare a wet-mount of the well-vortexed, heat-treated spore suspension (8.4.7) and observe at least 5 fields under a phase-contrast microscope. The spores will appear as oval and highly refractile structures while any vegetative cells would look like small rods or irregularly shaped debris. Count the numbers of spores and vegetative cells in each field, average the counts of the spores and vegetative cells separately, and calculate % purity of the spores using the formula:

% purity= (average spore count/ (average spore count + average vegetative cell count)) × 100

The level of spore purity should be ≥90%.

10.2 Subject a smear of the spore suspension to staining with the malachite green-safranin method (8). The spores will take up malachite green and the vegetative cells will show the reddish color of safranin.

11. Enumeration of Viable Spores

11.1 Perform serial tenfold dilutions of the spore suspension in PBS-T out to 10^{-7} for spread-plating or 10^{-9} for membrane filtration.

11.2 Spread plate with 0.1 mL of the appropriate dilutions on BHIYT-L in duplicate or filter the appropriate tenfold dilution through 47-mm diameter membranes with a pore diameter of 0.22 µm.

11.3 Incubate the plates anaerobically at 36 ± 1°C for 48 ± 4 h.

11.4 Record the numbers of CFU; the titer should be >10^9 viable spores/mL.

12. Quantitative Acid Resistance Test

12.1 Place 990 µL of 2.5 M HCl into each of three sterile 15-mL conical screw-caped tubes; for the control, place 990 µL of deionized water into one 15-mL conical screw-caped tube.

12.2 Using a 20-µL positive-displacement pipette, add 10 µL of the semipurified spore suspension from 8.4 (with a CFU titer of $>10^8$/mL) to the center of each tube while avoiding contact with the inside wall of the tube; this would give $>10^6$ CFU/tube. Vortex each tube.

12.3 Hold the acid-containing tubes and the controls for 10 min at room temperature (23 ± 1°C).

12.4 At the end of the holding period, add to each tube 9.0 mL of a 0.25 M NaOH to neutralize the acid. To the control tubes add 9.0 mL of a mixture of 1 mL of 2.5 M HCl and 9.0 mL of 0.25 M NaOH.

12.5 Dilute each spore suspension tenfold out to 10^{-6} in PBS and membrane filter only the last three dilutions (10^{-3} to 10^{-6}). Plate each filter separately on a plastic Petri dish containing BHIYT-L.

NOTE 3—Other means of titration, such as spread-plating, may also be used.

12.6 Invert plates and incubate for 48 ± 4 h at 36 ± 1°C under anaerobic conditions.

12.7 The spores are considered acid-resistant if the loss in their viability is <2 $\log_{10}$ as compared with the controls (9).

13. Precision and Bias

13.1 In a seven-laboratory collaborative (10) the average repeatability (within lab) and reproducibility (among labs) values of $\log_{10}$ densities of high-titered suspensions of viable semi-purified spores produced using the liquid medium determined.

13.2 The repeatability and reproducibility standard deviations were found to be 0.2 and 0.33, respectively.

14. Keywords

14.1 Anaerobic spore-former; *Clostridium difficile*; enzymatic purification; liquid medium for spore production; sporicidal activity

APPENDIX

(Nonmandatory Information)

X1. SEMIPURIFICATION OF THE SPORE SUSPENSION BY SUCROSE DENSITY GRADIENT (ALTERNATIVE METHOD)

X1.1 Instead of the enzymatic treatment method, the spores can be semi-purified using sucrose density gradient as described here.

X1.1.1 Make a 50% (w/v) solution of sucrose in water and sterilize by membrane (0.22-µm pore diameter) filtration or autoclaving.

X1.1.2 Pipette 5 mL of the sucrose solution in each of two 15-mL plastic conical tubes.

X1.1.3 Layer 1 mL of the spore suspension to be purified (up to 0.2 g of washed spores in 1 mL) on top of the sucrose solution in each tube.

X1.1.4 Centrifuge tubes at 3500×g for 10 min using a swinging-bucket rotor.

NOTE X1.1—Use of a swinging-bucket rotor is mandatory for this step.

X1.1.5 After centrifugation, remove (by vacuum aspiration) and discard the top layers without disturbing the bottom pellet.

X1.1.6 Collect the spores in the bottom pellet.

X1.1.7 Resuspend the pellet in 1 mL of PBS-T and transfer it to a 2-mL sterile cryovial or siliconized microcentrifuge tube.

X1.1.8 Centrifuge tube at 10 000×g for 3 min.

X1.1.9 Discard the supernatant and resuspend the pellet with 1-1.5 mL cold (2-5°C) PBS-T. Mix by vortexing to disaggregate the pellet. Repeat the washing step two more times.