Designation: E2895 – 19

Standard 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. Appendix X1 describes an alternative to enzyme purification.

1. Scope

1.1 This practice 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 E2111and E2197); such spores can also be used to study their structure, chemistry and germination.

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

1.7 *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]

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

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

[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.

Current edition approved April 1, 2019. Published May 2019. Originally approved 2019. Last previous edition approved 2013 as E2895–13. DOI: 10.1520/E2895-19

[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.

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 practice, 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 Practice

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 sucrose 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 practice may be used to assess environmental surface disinfectants for 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* ($NaH_2PO_4 \cdot H_2O$).

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* ($CaCl_2 \cdot 2H_2O$).

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).

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 ± 1 °C.

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.

[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] Trademark ICI Americas.

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 *Water bath,* 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, polyethersulfone (PES) membranes preferred.

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 *Clostridium difficile* ATCC 43598.

6.4.2 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:

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 121 °C.

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

7.1.2.5 Place the flasks in an anaerobic incubator (36 ± 1°C) for 18-24 h to pre-reduce 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 pre-reduced 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 pre-reduced 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 8.1.4 into each 50-mL conical tube containing 20 mL of pre-reduced 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:*