Designation: E3011 – 15

Standard Test Method for In vitro production of *Clostridium Difficile* Spores[1]

This standard is issued under the fixed designation E3011; 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 test method is designed to propagate spores of *Clostridium difficile* using liver broth.

1.2 It is the responsibility of the user of this test method to determine whether Good Laboratory Practices are required and follow when appropriate.

1.3 This test method should only be performed by those trained in microbiological techniques.

1.4 *Units*—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. Referenced Documents

2.1 *ASTM Standards:*[2]

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

E2895 Test Method for Producing High Titers of Viable and Semi-Purified Spores of Clostridium difficile using a Liquid Medium

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *Federal Standard:*[3]

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 Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *frozen stock culture, n*—a culture of vegetative bacteria propagated, and prepared for storage at ≤ –70°C in a liquid broth medium containing a cryoprotectant such as glycerol.

3.2.2 *spore suspension, n*—harvested spores suspended in a liquid medium, sterile deionized water.

4. Summary of Test Method

4.1 This standard outlines a procedure for producing high-titer spore suspensions of *C. difficile* using a commercially available liquid medium with 7 to 10 d of incubation under anaerobic conditions. Once adequate levels of spores are present in the liquid medium, the spores are harvested and washed several times in cold sterile deionized water. The spore suspension is enumerated, the spore purity is assessed and the acid resistance is verified.

5. Significance and Use

5.1 This test method describes a procedure for producing spore suspensions of *C. difficile* ATCC 700792, *C. difficile* ATCC 43598, or *C. difficile* ATCC 43599. The spore suspensions may be used in antimicrobial efficacy testing, or other laboratory testing requiring *C. difficile* spores. A spore crop is considered acceptable if the titer is >8 $\log_{10}$ spores/mL, purity of 95%, and is resistant to 2.5M HCl after 10 min of exposure (see Test Method E2839).

6. Apparatus

6.1 *Anaerobe jar*—Any airtight jar or container that can be used in combination with gas packs to obtain an anaerobic environment. An anaerobic chamber and anaerobic incubator may be substituted for an anaerobe jar.

6.2 *Biological safety cabinet*—To help maintain an aseptic work space.

6.3 *Centrifuge*—Any type or model capable of centrifuging up to 40 mL of liquid at 7500 × g.

6.4 *Centrifuge tubes*—Any type of sterile centrifuge tube with a 50 mL capacity.

6.5 *Incubator*—An incubator capable of maintaining 36±1°C.

6.6 *Laboratory glassware with closures*—Glassware to hold up to 1L of media, and withstands autoclave temperatures.

[1] This test method 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] Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) 40 CFR Part 160, Good 185 Laboratory Practice Standards; Final Rule. 1989.

Closures must be able to cover opening of glassware to protect media from environmental contamination.

6.7 *Microcentrifuge tubes*—Sterile 1.5 mL volume microcentrifuge tubes.

6.8 *Micropipettors*—Any suitable models capable of pipetting 10 µL or up to 1000 µL, or both. Calibrated micropipettors are preferred.

6.9 *Micropipette tips, sterile*—Any sterile micropipette tips for use with Micropipettors in 6.8.

6.10 *Microscope*—Any microscope capable of 1000× magnification with phase contrast options.

6.11 *Plate spreader*—Any sterile spreader for spreading inocula on agar plates.

6.12 *Serological pipettes*—Any sterile, single use pipettes that are capable of pipetting 1.0 mL, 10 mL, or 50 mL volumes.

6.13 *Sterile cheesecloth*—Two layers of cheesecloth is placed inside an appropriately sized funnel and sterilized.

6.14 *Vortex mixer.*

7. Reagents and Materials

7.1 *Liver Broth*[4]—Used to sporulate C. difficile.

7.2 *Butterfield's Phosphate Buffer Stock Solution, 0.25M (PBSS)*—Dissolve 34.0 g of monobasic potassium phosphate in 500 mL of deionized water. Adjust pH to 7.2 with 10N NaOH, and dilute to 1 L.

7.3 *Butterfield's Phosphate Buffered Dilution Water (PBDW)*—Add 1.25 mL of 0.25M PBSS to 1 L deionized water. Dispense into 9 mL or 99 mL portions. Autoclave for 20 min at 121°C.

7.4 *pH adjusted Phosphate Buffered Dilution Water (pH adjusted PBDW)*—PBDW with the pH adjusted with sterile 1 M Sodium hydroxide (NaOH) to a pH that neutralizes 2.5 M Hydrochloric Acid (HCl) (pH 11-12 is suggested). The pH of PBDW may also be adjusted with non-sterile 1 M NaOH prior to autoclave sterilization, provided the pH after sterilization is adequate to neutralize 2.5 M HCl.

7.5 *Recovery Medium for Enumeration of Spore Suspension*—Brain Heart Infusion Agar with yeast extract (5 g/L), horse blood (70 mL/L) and sodium taurocholate (1 g/L) (BHIY-HT) , pre-reduced.

7.6 *Hydrochloric acid (HCl)*—2.5 M HCl is prepared from 5 M HCl.

7.7 *Water*—Sterile deionized water.

8. Hazards

8.1 *C. difficile* is a Biosafety Level 2 organism. Appropriate safety procedures, as recommended by the US Centers for Disease Control and Prevention/National Institutes of Health[5] or other local agency, should be used with this organism.

8.2 Consult Material Safety Data Sheets (MSDS) for the chemicals used in this method to determine the appropriate personal protective equipment required for handling each chemical.

9. Test Organism

9.1 Frozen stock cultures of *C. difficile* ATCC 700792, *C. difficile* ATCC 43598, or *C. difficile* ATCC 43599. Frozen stock cultures may be prepared from cultures obtained from a reputable vendor or culture collection agency.

9.2 Other strains of *C. difficile* may be sporulated using this method.

10. Procedure

10.1 *Sporulation of Clostridium difficile in Liquid Medium:*

10.1.1 Inoculate 1 L of Liver Broth with 0.25 mL to 0.5 mL of frozen stock culture of *C. difficile*. Other volumes may be used as long as the ratio of Liver Broth to frozen stock culture is the same as previously stated.

10.1.2 Incubate Liver Broth under anaerobic conditions for 36±1°C for 7-10 d, or until at least 95% spores are present. Some strains of *C. difficile* may need more than 10 d to achieve the desired level of sporulation.

10.1.3 Use phase contrast microscopy, at 1000× magnification, to determine percent of spores present in the Liver Broth. Stir Liver Broth prior to preparing slide for phase contrast microscopy. Count spores and vegetative cells in five fields of view. The broth is ready to be harvested when the percent spores is ≥95% as determined using equation in 11.1. Checking the spore purity beginning around d 7 is recommended.

10.1.4 Stir Liver Broth to resuspend any spores that have settled to the bottom. Filter the entire volume of Liver Broth through sterile cheesecloth, and collect in sterile centrifuge tubes.

10.1.5 Centrifuge filtered broth at 7500 × g for 20 mins at 20 ± 2°C. Dispose of the supernate and resuspend with filtered broth until all broth has been centrifuged. Resuspend the final pellets in 20 to 30 mL of sterile deionized water.

10.1.6 Wash the final pellets four times by centrifuging at 7500 x g for 20 mins at 20 ± 2°C and discard supernate. Resuspend the pellet in sterile cold (2 - 8°C) deionized water.

10.1.7 Resuspend the final pellet in 10 to 30 mL of sterile cold deionized water to achieve the desired concentration of spores. Store spore suspension at 2 - 8°C for up to 6 months.

10.1.8 Use phase contrast microscopy, at 1000× magnification, to determine percent of purity of the spore suspension. Mix the spore suspension well. Count spores and vegetative cells in five fields of view. The spore suspension should have ≥95% spores as determined using equation in 11.1.

10.1.9 Serially dilute the spore suspension in PBDW. Spread plate appropriate dilutions in duplicate on BHIY-HT.

[4] The sole source of supply for the Liver Broth (Cat. No. M928-500G) known to the committee at this time is HiMedia Laboratories, Marg, Mumbai, India. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Composition of media available at www.himedialabs.com/TD/M928.pdf accessed on February 23, 2015.

[5] Centers for Disease Control and Prevention, and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories, 5th ed., United States Department of Health and Human Services, Washington, DC, December 2009.

Incubate inverted plates at 36±1°C for 72 ± 4 h under anaerobic conditions. Determine the $\log_{10}$ of the average CFU/mL of the spore suspension.

10.1.10 The spore suspension may be diluted or concentrated as needed for efficacy testing.

10.2 *Quantitative Acid Resistance Test*—HCl Resistance (See Test Method E2839.)

10.2.1 Place 990 µL of 2.5 M HCl into three microcentrifuge tubes. Place 990 µL of sterile deionized water into one microcentrifuge tube.

10.2.2 Using a micropipettor, preferably a positive displacement pipette, add 10 µL of the high titer (>8 $\log_{10}$ spores per mL) spore suspension to each microcentrifuge tube prepared in 10.2.1. Vortex each tube. The microcentrifuge tubes should be held at ambient temperature during the exposure time.

10.2.3 At the end of the 10 min exposure time for the HCl microcentrifuge tubes, transfer 0.1 mL from one tube to a microcentrifuge tube with 900 µL of pH adjusted PBDW to neutralize. 0.1 mL from the control microcentrifuge tube is transferred to a tube of 900 µL of pH adjusted PBDW after a 10 min exposure time.

10.2.4 Serially dilute each neutralized spore suspension in PBDW and spread plate 0.1 mL from appropriate dilutions in duplicate onto BHIY-HT. Incubate inverted plates at 36±1°C for 72 ± 4 hours under anaerobic conditions.

10.2.5 A spore suspension is considered to be acid resistant if the Log_{10} reduction is between 0 and 2 at 10 mins of exposure as compared to the control.

11. Calculation or Interpretation of Results

11.1 *Percent Spores Present:*

$$\text{Precent Spores Present} = \frac{\text{Average Spore Count}}{\text{Average Spore Count} + \text{Average Vegetative Cell Count}}(100) \quad (1)$$

11.2 *Log_{10} of the Average CFU/mL of the Spore Suspension:*

$$\text{Average CFU/mL} = \frac{(\text{Mean CFUper plate})(\text{Reciprocal of dilution})}{\text{Volume Plated}}(100) \quad (2)$$

11.3 *Log_{10} Reduction of HCl treatment:*

Log Reduction = LC – LH

LC = Log_{10} of viable spores after control treatment

LH = Log_{10} of viable spores after HCl treatments

12. Precision and Bias

12.1 A precision and bias statement cannot be made for this test method at this time.

13. Keywords

13.1 *C. difficile*; *Clostridium*; liver broth; propagation; spores; spore crop; sporulation