US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for Production and Storage of Spores of *Clostridioides difficile* for Use in the Efficacy Evaluation of Antimicrobial Agents

SOP Number: MB-28-08

Date Revised: 09-20-22

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Revisions Made	• Minor editorial changes for clarification purposes. • Added language in carriers section; the top of the disk is brushed and has round edges (Section 11.1.q.i). • When preparing mucin, it is now passed through a 0.2 µm pore diameter membrane filter instead of autoclaved (Section 11.2.1.iii). • Removed footnotes stating additional confirmatory procedures not prescribed in ASTM E2839-18. • Updated ASTM International Standard reference from 2018 to 2021 and updated version number from E2839-18 to E2839-21.

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Title	Production and Storage of Spores of *Clostridioides difficile* for Use in the Efficacy Evaluation of Antimicrobial Agents
Scope	This document specifies the procedures for producing and storing standardized suspensions of *Clostridioides difficile* spores based on ASTM E2839-21.
Application	For use in the evaluation of 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.

	Approval	Date
SOP Developer	Lisa Smith	09/20/22
	Print Name: Lisa Smith	
SOP Reviewer	Rebecca Pines	09/20/22
	Print Name: Rebecca Pines	
Quality Assurance Unit	Michele Cottrill	09/20/22
	Print Name: Michele Cottrill	
Branch Chief	Rebecca Pines	09/20/22
	Print Name: Rebecca Pines	

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1. Definitions	1. Additional abbreviations/definitions are provided in the text. 2. Pre-reduced medium: an agar or broth manufactured in an oxygen-free environment and packaged in air-tight sealed pouches or bags. 3. Density gradient medium: HistoDenz™ is a non-ionic gradient medium used here to separate spores from vegetative cells and cell fragments on the basis of density. 4. Purified spores: level of quality based on when the spore concentration reaches ≥95%, as vegetative cells and cell fragments are separated by the density gradient medium. 5. Toxigenic strain: possesses either toxin A gene (*tcdA*+) or toxin B gene (*tcdB*+) or both.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with chemicals.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-01 (pH meters), EQ-02 (thermometers and hygrometers), EQ-03 (weigh balances), EQ-05 (timers), and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	Not applicable
6. Quality Control	1. For quality control purposes, document the required information on the appropriate form(s) (see section 14). 2. Refer to SOP MB-10, Media and Reagents Preparation and Quality Evaluation, for QC of media and reagents.
7. Interferences	The test organism must be incubated under strict anaerobic conditions. If an anaerobic environment is not maintained, the presence of oxygen will compromise the viability of *C. difficile*.
8. Non-conforming Data	1. The acceptance criteria for a spore suspension are: (1) spore titer of approximately 5.0×10^8 spores/mL, (2) spore purity of ≥95%, and (3) a mean $\log_{10}$ reduction (LR) value >5.0 for 3 carriers exposed to 5,000 ppm and a mean LR of <3.0 for 3 carriers exposed to 1,500 ppm sodium hypochlorite. These acceptance criteria are necessary in order to use the spore suspension to evaluate the performance of antimicrobial formulations using SOP MB-31, Quantitative Method for

	Testing Antimicrobial Agents against Spores of *C. difficile* on Hard, Non-porous Surfaces. 2. Management of non-conforming data will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Archive the data consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Seal culture plates with Parafilm™, or equivalent, to prevent dehydration during the extended anaerobic incubation. 2. Ensure media (e.g., Reinforced clostridial medium) is pre-reduced for at least 24±2 h prior to use. 3. Place inoculated plates under anaerobic conditions within 1 hour of opening the sealed package of plates.
11. Special Apparatus and Materials	1. Apparatus. a. *Calibrated micropipettes* (e.g., 200 µL, 1000 µL). 1000 µL pipettes with corresponding tips for transferring reagents and spores. 200 µL pipettes with corresponding tips for deposition of test substance on carrier. b. *Sterile centrifuge tubes*. Polypropylene, 15 mL and 50 mL graduated plastic centrifuge tubes with conical bottoms. c. *Microcentrifuge tubes*. Siliconized, sterile 1.5-mL low-retention microcentrifuge tubes. Use for dilutions and processing of spores during purification. d. *Centrifuge with swinging-bucket rotor*. To allow sedimentation of spores for washing and/or concentration. e. *Microcentrifuge*. To allow sedimentation of spores for washing and/or concentration. f. *Inoculating loop*. 10 µL loop to streak plates. g. *Vortex mixer*. h. *Polyethersulfone membrane filter* (PES). For recovery of test spores, 47 mm diameter and 0.2 µm pore size. Use any filtration apparatus including filtration units (reusable or disposable). i. *Anaerobic chamber*. Supported by a gas mixture containing 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.

j. *Incubator*. Use an incubator at 36±1°C inside an anaerobic chamber to support the growth of the organism. Alternatively, place the anaerobic jars in an aerobic incubator at 36±1°C.

k. *Microscope with 10X eyepiece and 100X (oil) objective with phase contrast option.* To evaluate purity of spore suspension.

l. *Timer*. Any timer that can display time in seconds.

m. *Cell scraper/spreader*. To aid in the removal of spores from agar plates.

n. *Laboratory film (or sealable bag)*. To seal inoculated plates during extended incubation (>48 h).

o. *Refrigerator (2-8°C)*. For short term storage of spore suspension during the purification process.

p. *Freezer (-80±5°C)*. For long term storage of stock cultures and spore suspensions.

q. *Carriers.* For use in spore qualification using NaOCl.

i. Disks (1 cm in diameter) made of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side (top). The top of the disk is brushed and has rounded edges; only the top is visually screened and inoculated. Carriers are single-use only. See MB-31 for complete carrier specifications and photographs of screened carriers.

r. *Calibrated 10 µL positive displacement pipette with corresponding 10 µL tips*. For carrier inoculation.

s. *Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes*. For rinsing vials and filters.

t. *Sterile forceps.* To handle membrane filters and to pick up the carriers for placement in vials.

u. *Filter paper*. 150 mm diameter, to line Petri plates.

v. *Sterile vials with lids (plastic or comparable)*. For holding inoculated carriers to be exposed to the test chemical and for accommodating 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.

w. *Desiccator (with gauge to measure vacuum) with fresh desiccant (e.g., $CaCO_3$)*. For drying the inoculum on the carriers.

x. *Vacuum source*: In-house line or suitable vacuum pump (20-25 in mercury, 0.068 to 0.085 MPa) for drying carriers and for filtering.

y. *Digital titrator kit*. To measure total chlorine. Alternate titration methods may be used.

2. Media and Reagents

a. *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 at least 24±2 h prior to use.

b. *RCM plus 15% glycerol (cryoprotectant)*. For use as the 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±2 h prior to use.

c. *CDC anaerobic 5% sheep blood agar (CABA)*. Sporulation medium commercially available pre-reduced (e.g., Anaerobe Systems, Morgan Hill, CA, or equivalent).

d. *Recovery media for enumeration of viable spores.* Pre-reduced brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT), commercially available pre-reduced (e.g., Anaerobe Systems, Morgan Hill, CA, or equivalent).

e. *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.

f. *PBS containing 0.1% (v/v) Tween 80 (PBS-T)*. Diluting and washing reagent; PBS-T pH is 7.2±0.2.

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

h. *Water.* Use either deionized distilled water or water with equivalent quality for making reagent solutions and culture media.

i. *Reagent-grade sodium hypochlorite (NaOCl) with total chlorine ≥4%.* Use to prepare 5,000±250 ppm and 1,500±150 ppm total chlorine solutions to qualify spores.

	j. *Tween-80* (polysorbate 80). Use to prepare PBS-T. k. *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. l. *Soil load.* Use in assay to qualify spore suspension. Soil load is a mixture of the following stock solutions: i. 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. ii. 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. iii. 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. iv. Aseptically aliquot soil stock solutions and store for up to one year at -20±5°C. The stock solutions of the soil load are single use only; do not refreeze once thawed. Other volumes of the stock solutions may be prepared following the same ratio. 2. Test Organism a. *Clostridioides difficile* (ATCC 43598), formerly known as *Clostridium difficile*, a toxigenic strain (*tcdA*-, *tcdB*+), obtained from a reputable vendor. The strain produces only Toxin B (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±1ºC.
12. Procedure and Analysis	In brief, the method provides detailed instructions for the culture, maintenance, sporulation and storage of *C. difficile* spores. Purified spores are enumerated and assessed for quality using a carrier-based test employing two concentrations of sodium hypochlorite (NaOCl).
12.1 Preparation of Frozen Stock Cultures of Test	a. *C. difficile received in lyophilized vegetative form:* i. In an anerobic environment, reconstitute contents of the lyophilized culture with 0.5 mL of sterile pre-reduced

Organism (Vegetative Form)	RCM according to the manufacturer's instructions. ii. After rehydration, aseptically transfer the vial contents to a tube containing 4±1 mL of pre-reduced RCM and mix by gentle vortexing. b. *C. difficile received as frozen vegetative culture:* i. Thaw frozen culture at room temperature. ii. In an anaerobic environment, transfer the contents to a tube containing 4±1 mL of sterile pre-reduced RCM and mix by gentle vortexing. c. *Inoculation of CABA Plates for Vegetative Stock Culture:* i. Spread plate 100 µL of the reconstituted culture in RCM on each of five CABA plates. ii. Streak one CABA plate for isolation to check for culture purity. iii. Invert plates and incubate anaerobically at 36±1°C for 48±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. d. *Harvest of CABA Plates for Stock Culture:* i. Following incubation (12.1.c.iii), add approximately 2 mL of sterile and cryoprotectant (11.2.b) to each CABA plate. ii. 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. iii. Pool the suspensions, mix thoroughly, and pipette approximately 0.4±0.1mL aliquots into cryovials; cap tightly. iv. Store the cryovials at −80±5°C. These vials contain the Vegetative Frozen Stock (VFS) Culture. e. *Evaluation of Vegetative Frozen Stock:* i. Within 2-7 days after freezing, thaw a VFS cryovial at room temperature, preferably under anaerobic conditions. If processing under aerobic conditions, perform steps 12.1e, i – 12.1e, iv within 15 min.

	ii. Vortex suspension thoroughly for at least 30±5 s and serially dilute 0.1 mL out to 10^{-6} in PBS-T. iii. Spread-plate 100 µL from 10^{-5} and 10^{-6} dilution tubes in duplicate on BHIY-HT. iv. Invert plates and incubate anaerobically at 36±1°C for 48±4 h. Record the number of colony forming units (CFU) per 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 cultures for a maximum of 18 months then reinitiate using a new lyophilized culture. v. Note: New VFS cultures may be initiated one time using an existing, unexpired frozen stock culture.
12.2 Preparation of a test spore suspension from VFS	a. *Inoculation of CABA plates.* Thaw a VFS cryovial at room temperature. Streak three CABA plates with the VFS, preferably under anaerobic conditions. i. Initiate anaerobic incubation for two plates and aerobic incubation for one plate at 36±1°C for 48±4 h within 15 min of removing the VFS cryovial from storage. ii. 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. Record observations on the *C. difficile* Spore Suspension Initiation Processing Sheet (see section 14). b. 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±1°C for 24±2 h. c. 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 disposable sterile spreader to create a lawn. d. 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±l°C and approximately 70% relative humidity. Maintenance of relative humidity is not required if an anaerobic jar is used. i. 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. Record observations. e. 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 **Attachment 1**); if below approximately 90%, discard the inoculated plates and initiate a new culture (12.2.a). f. *Harvesting CABA plates.* 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. g. 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. h. *Washing the spore suspension by centrifugation.* Centrifuge tubes from 12.2g at 4,500×g for 15 min. i. 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 4,500×g for 15 min. j. 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 4,500×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 4,500×g for 15 min. k. 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: 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.
	l.	*Heat treatment.* Heat the spore suspension in a heat block for l0±1 min at 65±2°C. To ensure that the spore suspension has reached 65±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 tube has reached 65±2°C.
	m.	Following the heat treatment, allow the suspension to cool to room temperature.
	n.	*Microscopic evaluation of spore suspension.* Mix the spore suspension (12.2.m) 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%.
	o.	*Evaluate titer of the spore suspension.* Mix the spore suspension vigorously by vortexing (30±5 s) prior to taking an aliquot of the spore suspension (e.g., 10 µL). Dilute the spore suspension out to 10^{-7} in PBS-T.
	p.	Spread-plate 0.1 mL of the 10^{-6} and 10^{-7} dilutions on BHIY-HT in duplicate.
	q.	Once the inocula have dried, invert plates and incubate anaerobically at 36±1°C for 48±4 h. Record the number of CFU/plate. The titer should be $>10^{8}$ spores/mL. Store spore suspension at 2-8ºC for up to 5 days prior to purification.
	r.	Streak one BHIY-HT plate for isolation to check for culture purity of the spore suspension. Invert plate and incubate anaerobically at 36±1°C for 48±2 h along with the titer plates. Observe BHIY-HT plate for purity, colony morphology, and characteristics of *C. difficile*.
12.3 Spore Purification	a.	Prepare a 50% (w/v) solution of HistoDenz™ in sterile deionized water (11.2.k) and pipet 5 mL into each of four sterile 15 mL plastic conical tubes. Bring the HistoDenz™ and the spore preparation to room temperature before use.
	b.	Layer 1 mL of spore suspension (12.2.m) on top of each of the four tubes of HistoDenz™. Centrifuge tubes at 4,500×g for 10

	min using a swinging bucket rotor. Use of a swinging bucket rotor is essential for proper layer removal and spore retention. c. Following centrifugation, three distinct layers should be present in the HistoDenz™. Using a 1 mL pipet, carefully remove the top three layers: 1) an upper clear layer, 2) a dense (opaque) second layer, and 3) a cloudy third layer. Discard the top three layers, leaving the pellet and the 3 to 4 mm cloudy layer above the pellet undisturbed. d. Using a repetitive pipetting action, resuspend and mix the pellet (without touching the pellet) with a 1 mL micropipette. Bring the volume up to approximately 1 mL with sterile cold (2-8°C) PBS-T. e. Mix thoroughly by vortexing (30±5 s) to break up the pellet (ensure absence of visual clumps or fragments of the pellet) and transfer the contents of each tube to siliconized microcentrifuge tubes. f. Centrifuge the microcentrifuge tubes (four total tubes) at 16,000×g for 5 min. Discard the supernatant and resuspend the pellets in 1 mL of sterile, cold (2-8°C) PBS-T. g. Cap the tubes and mix by thoroughly vortexing (30±5 s) to break up the pellets (ensure absence of visual clumps or fragments of the pellet). h. Centrifuge the microcentrifuge tubes at 16,000×g for 2 min. Discard the supernatant and resuspend the pellets in 1 mL of sterile, cold (2-8°C) PBS-T. Cap the tubes and mix thoroughly by vortexing to break up the pellets (ensure absence of visual clumps or fragments of the pellet). This step is the first wash. i. Repeat washing procedure (12.3.h) two additional times, for a total of three washes. j. After the third wash, discard the supernatant and resuspend the pellets in each microcentrifuge tube with 0.5 mL of sterile, cold (2-8°C) PBS-T. Pool the contents of each microcentrifuge tube into one 15 mL conical tube. This is the purified spore suspension. The purified spore suspension may be stored at 2-8°C for up to 10 days prior to freezing. k. Determine spore purity using procedures stated in 12.2n and calculate purity of the spore suspension using the formula

	presented in section 13.1. The purity of spores should be ≥95% (see **Attachment 2**). l. Determine titer of the purified spore suspension as in 12.2o-q; use the formula presented in 13.2 for calculations. The purified spore suspension should be approximately 10^9 spores/mL. i. Based on the titer calculated in 12.3k, it is recommended to dilute a small aliquot (e.g., 100 µL) of the purified spore suspension with sterile PBS-T to achieve a concentration of approximately 5.0×10^8 spores/mL. Determine titer of spores from the diluted aliquot by making serial dilutions of the aliquot out to 10^{-6} and plate 0.1 mL from 10^{-5} and 10^{-6} dilutions on BHIY-HT plates in duplicate. Incubate plates anaerobically for 48±4 h at 36±1°C. Record the number of CFU and calculate the titer. m. Dilute the purified spore suspension targeting a titer of 5.0×10^8 spores/mL. n. Determine the titer of the diluted purified spore suspension from 12.3m. Attain a titer of approximately 5.0×10^8 spores/mL. o. Streak one BHIY-HT plate for isolation to check for culture purity of the purified spore suspension. Invert plate and incubate anaerobically at 36±1°C for 48±4 h along with the titer plates. Observe BHIY-HT plates for purity, colony morphology, and characteristics of *C. difficile*. For confirmatory purposes, conduct biochemical and antigenic analyses or other comparable confirmation procedures (e.g., Vitek).
12.4 Long Term Spore Storage	a. Assign a preparation number to the final spore suspension. b. Freeze spores within 10 days of purification (step 12.3j). Aliquot the diluted purified spore suspension into cryovials (~0.5 mL/vial) and store at -80±5°C. Store/use frozen spores for up to 90 days. Each cryovial is for single use only.
12.5 Spore Qualification	a. Within 2-10 days of storage, qualify the frozen spore suspension by conducting the Quantitative Method for Testing Antimicrobial Agents against Spores of *C. difficile* on Hard, Non-porous Surfaces (SOP MB-31) using two concentrations of reagent grade NaOCl. b. Using sterile deionized water as the diluent, prepare 5,000±250 ppm and 1,500±150 ppm solutions of NaOCl.

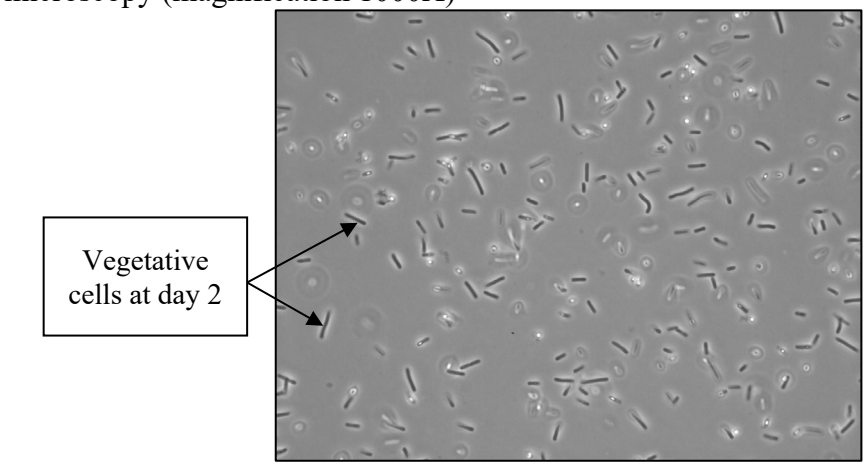
	c. Verify the concentration of the NaOCl solutions using an appropriate titration procedure prior to use. Use NaOCl solutions within 3 h of preparation. d. Refer to sections 12.2 and 12.3 of SOP MB-31 for preparation of final spore suspension (with three-part soil load) and inoculation and drying of carriers, respectively. e. Inoculate a minimum of eleven carriers (e.g., three for each concentration of NaOCl, three for control, and two extras). f. Use sterile PBS-T with 0.1% (w/v) sodium thiosulfate as the neutralizer and a contact time of 3 min ±5 s at room temperature (22±2°C). i. Confirm the effectiveness of the neutralizer (PBS-T with 0.1% (w/v) sodium thiosulfate) against 5,000 ppm NaOCl using the procedure in SOP MB-31. g. The mean $\log_{10}$ density for control carriers is 6.0-7.0 spores/carrier. h. Serially dilute the 10^0 tube (vial containing the carrier) for each treatment (e.g., out to 10^{-1} for 5,000 ppm and out to 10^{-5} for 1,500 ppm) and filter the following dilutions: i. 5,000 ppm: 10^0 and 10^{-1} ii. 1,500 ppm: 10^{-3}, 10^{-4} and 10^{-5} i. Record the appropriate number of CFU per filter (e.g., up to 200 CFU) and calculate the $\log_{10}$ reduction. j. The spore suspension is qualified if the following $\log_{10}$ reduction values are observed: i. 5,000 ppm: >5.0 ii. 1,500 ppm: <3.0 iii. If these LR values are not observed, the spores are not deemed acceptable for testing.
12.6 Acceptance criteria	a. The spore suspension is acceptable for use if all required criteria have been met: i. Spore titer of approximately 5.0×10^8 spores/mL. ii. Mean spore purity of ≥95%. iii. Mean LR against two concentrations of NaOCl (>5.0 for 5,000 ppm and <3.0 for 1,500 ppm).

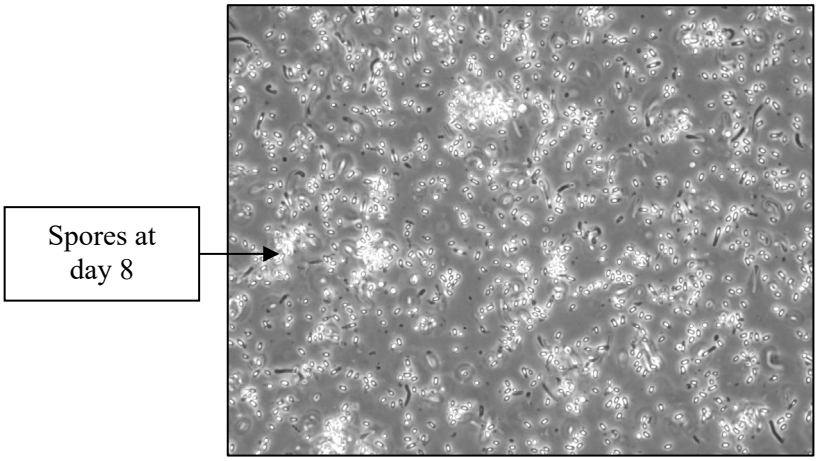
13. Data Analysis/ Calculations	1. Determine spore suspension purity: $$\%\ Purity = 100\ \% \times \frac{A}{A+B}$$ Where *A* = mean spore count, and *B* = mean vegetative cell count. 2. Determine the titer of vegetative cultures and spore suspensions (CFU/mL): $$\text{Titer as CFU/mL} = \frac{\text{A} \times \text{B}}{\text{C}}$$ Where *A* = mean colony count at dilution plated, *B* = reciprocal of dilution used, and *C* = volume plated.
14. Forms and Data Sheets	1. Attachment 1: Sporulation of *C. difficile*. 2. Attachment 2: Purified *C. difficile* spores. 3. Test Sheets: Test sheets are stored separately from the SOP under the following file names: *C. difficile* Spore Titer Form — MB-28-08_F1.docx Organism Culture Tracking Form — MB-28-08_F2.docx Test Microbe Confirmation Sheet (Quality Control) — MB-28-08_F3.docx *C. difficile* Vegetative Frozen Stock Culture Processing Sheet — MB-28-08_F4.docx *C. difficile* Spore Suspension Initiation Processing Sheet — MB-28-08_F5.docx *C. difficile* Spore Suspension Harvesting Processing Sheet — MB-28-08_F6.docx *C. difficile* Spore Suspension Purification Processing Sheet — MB-28-08_F7.docx QM for *C.d.*: Test Information Sheet — MB-31_F1.docx QM for *C.d.*: Time Recording, Dilution and Filtration Sheet — MB-31_F2.docx QM for *C.d.*: Results Sheet — MB-31_F3.docx QM for *C.d.*: Test Microbe Confirmation Sheet — MB-31_F4.docx QM for *C.d.*: Processing Sheet — MB-31_F5.docx
15. References	1. ASTM E2839-21, Standard Practice for Production and Storage of

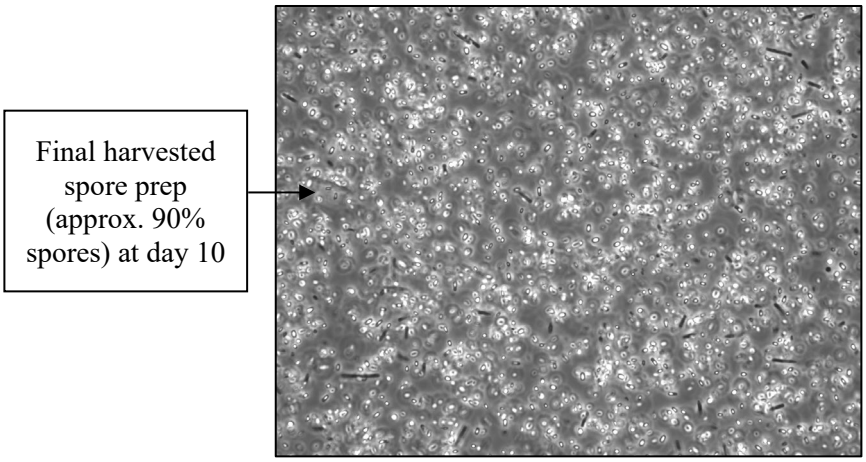
	Spores of *C. difficile* for Use in Efficacy Evaluation of Antimicrobial Agents. ASTM International, West Conshohocken, PA, 2021. 2. Hasan, J. A., Japal, K. M., Christensen, E. R. and Samalot-Freire, L. C., "Development of methodology to generate *Clostridium difficile* spores for use in the efficacy evaluation of disinfectants, a pre-collaborative investigation," *J. AOAC Int*, Vol 94, 2011, pp. 259-272.

Attachment 1

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







Attachment 2

Purified *C. difficile* spores (ATCC 43598), using HistoDenz™ (≥95% purity; magnification 1000X)