US Environmental Protection Agency
Office of Pesticide Programs

EPA MLB SOP MB-28: Procedure for the Production and Storage of Spores of *Clostridium difficile* for Use in the Efficacy Evaluation of Antimicrobial Agents

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Production and Storage of Spores of *Clostridium difficile* for Use in the Efficacy Evaluation of Antimicrobial Agents

I. Overview

A. This document describes the test methodology for producing and storing standardized spore suspensions of *C. difficile* (ATCC 43598).

B. This method is based on ASTM E2839, Standard Test Method for Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents, ASTM International.

C. In brief, the method provides detailed instructions for the culture, maintenance, sporulation and storage of *C. difficile* spores. Spores are harvested from an agar medium following incubation under anaerobic conditions for 10 days. Upon harvesting, spores are washed several times with PBS-Tween 80, exposed to heat to inactivate any viable vegetative cells, and purified using a density gradient medium. Purified spores are enumerated and assessed for quality using a carrier-based test employing two concentrations of sodium hypochlorite (NaOCl).

D. Refer to EPA MLB SOP MB-31 [OECD Quantitative Method for Testing Antimicrobial Products Against Spores of *Clostridium difficile* (ATCC 43598) on Inanimate, Hard, Non-porous Surfaces] for materials and methods for conducting the carrier-based NaOCl testing to qualify spores for use.

II. Data Requirements

A. The acceptance criteria for the spore suspension are: (1) Spore purity of ≥ 95%; (2) Spore titer of approximately 5.0×10^8 viable spores/mL; (3) Log reduction (LR) values against two concentrations of NaOCl: a mean LR of >5 for 3 carriers exposed to 5,000 ppm and a mean LR of <3 for 3 carriers exposed to 1,500 ppm total chlorine.

III. Special Apparatus and Materials

A. Test microbe: *Clostridium difficile* (ATCC 43598), a toxigenic strain (*tcdA*-, *tcdB*+), 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 when grown anaerobically on the surface of CDC anaerobic 5% sheep blood agar (CABA) plates within 48 h at 36±1ºC.

B. Culture media

1. *Reinforced clostridial medium (RCM)*. Use to rehydrate 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.

2. *RCM plus 15% glycerol*. 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.

3. *CDC anaerobic 5% sheep blood agar (CABA) plates*. Use for sporulation medium, commercially available as pre-reduced (e.g., Anaerobe Systems, Morgan Hill, CA, or equivalent).

4. *Brain heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT)*. Use for recovery medium for enumeration of viable spores, commercially available as pre-reduced (e.g., Anaerobe Systems, Morgan Hill, CA, or equivalent).

C. Reagents

1. *Phosphate buffered saline stock solution (e.g., 10X).* Use to prepare 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2.

2. *Phosphate-buffered saline (PBS), 1X*. Use to prepare PBS containing 0.1% (v/v) Tween 80 (PBS-T) and PBS-T with 0.1% (w/v) sodium thiosulfate. 1X PBS with a pH of approximately 7.0±0.5 is desirable.

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

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

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

6. *Laboratory 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.

7. *Tween-80* (polysorbate 80). Use to prepare PBS-T.

8. *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.

9. *Soil load.* The standard soil load to be incorporated in the qualified spore suspension is a mixture of the following stock solutions:

   i. Bovine Serum Albumin (BSA): Add 0.5 g BSA to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot, and store at -20±5°C.

   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, aliquot, and store at -20±5°C.

   iii. Mucin: Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS, mix thoroughly until dissolved, and autoclave (15 min at 121°C), aliquot, and store at -20±5°C.

iv. The stock solutions of the soil load are single use only. Do not refreeze once thawed; store up to one year at -20±5°C.

10. *Liquinox (1% solution) or equivalent.* Use to clean carriers.

D. Apparatus

1. *Calibrated micropipettes* (e.g., 200 µL, 1000 µL). 1000 µL pipettes with 100-1000 µL tips for transferring reagents and spores. 200 µL pipettes with 10-100 µL or 20-200 µL tips for deposition of test substance on carrier.

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

3. *Microcentrifuge tubes*. Sterile 1.5-mL low-retention (siliconized). Use for dilutions and processing of spores during purification.

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

5. *Microcentrifuge*.

6. *Inoculating loop* (10 µL). To streak plates.

7. *Vortex mixer*.

8. *Polyethersulfone membrane filter* (PES). For recovery of test microbe, 47 mm diameter and 0.2 µm pore size. Use any filtration apparatus including filtration units (reusable or disposable).

9. *Anaerobic chamber*. Supported by a gas mixture containing at least 5% H_2 with the balance comprised of 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. Use to ensure anaerobic environment.

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

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

12. *Certified timer*. Readable in minutes and seconds.

13. *Cell scraper/spreader*. Use to remove spores from plates.

14. *Parafilm™* or sealable bag to seal inoculated plates during extended incubation (>48 h).

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

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

17. *Carriers:* Disks (1 cm in diameter) made from 0.8 mm thick sheets of brushed and magnetized stainless steel (AISI #430) (Pegen Industries, part #430-107) for use in spore qualification using NaOCl. 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 carrier specifications.

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

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

20. *Forceps*, straight or curved, non-magnetic, disposable with smooth flat tips to handle membrane filters. Use to pick up the carriers for placement in vials.

21. *Filter paper*. Whatman No. 2, to line Petri plates.

22. *Magnet.* Use to hold the carrier in place in the vial while the liquid is being dispensed into filter.

23. *Sterile vials* (plastic or comparable) to hold test carriers: flat bottom and wide-mouth to accommodate addition and removal of the carriers. For holding inoculated carriers to be exposed to the test substance and for accommodating neutralizer/eluent. Use vials at least 25 mm in neck diameter and capable of holding at least 20 mL of liquid.

24. *Desiccator* with fresh desiccant (e.g., $CaCO_3$). For drying the inoculum on the carriers.

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

26. *HACH's Digital Titrator kit*. To measure total chlorine. Alternate titration methods may be used.

IV. Procedure and Analysis

Preparation of Frozen Stock Cultures of Test Organism

A. *C. difficile* received in lyophilized vegetative form

1. To initiate a new stock culture, reconstitute contents of the lyophilized culture with 0.5 mL of sterile pre-reduced RCM in the anaerobic chamber as per the manufacturer's instructions.

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

B. *C. difficile* received as frozen vegetative culture

1. To initiate a new stock culture, thaw frozen culture at room temperature. Transfer the contents to a tube containing 4±1 mL of sterile pre-reduced RCM in the anaerobic chamber, and mix by gentle vortexing.

C. Inoculation of CABA plates with culture reconstituted in RCM

1. Spread plate 100 µL of the reconstituted culture in RCM on five CABA plates. Streak one CABA plate for isolation to check culture purity. Invert plates and incubate anaerobically at 36±1°C for 48±4 h.
2. Observe CABA plate for purity and colony morphology and characteristics of *C. difficile;* discard the CABA plates if not pure; re-initiate a new stock culture.
3. Add 2 mL of RCM plus 15% (v/v) glycerol to each of the five CABA plates.
4. Using a sterile cell scraper/spreader, gently scrape culture from the surface of the plate, aspirate with a pipette and transfer to a 15 mL conical tube
5. Repeat this process for the remaining four plates. Pool the suspensions, mix thoroughly, and pipette approximately 0.4±0.1mL aliquots into cryovials; cap tightly.
6. Store the cryovials at −80±5°C. These vials contain the Vegetative Frozen Stock (VFS) Culture.
7. Within 2-7 days of freezing, thaw a VFS cryovial at room temperature, preferably under anaerobic conditions. Vortex suspension thoroughly for at least 30±5 s and serially dilute 0.1 mL out to 10^{-6} in PBS-T. Spread-plate 100 µL from10^{-5} and 10^{-6} dilution tubes in duplicate on BHIY-HT. Invert plates and incubate anaerobically at 36±1°C for 48±4 h.
8. 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 a maximum of 18 months then reinitiate using a new lyophilized culture.
    i. Note: New VFS cultures may be initiated one time using an existing, unexpired frozen stock culture.

D. Preparation of a spore suspension from VFS

1. Thaw a VFS cryovial at room temperature. Streak three CABA plates with the VFS, preferably under anaerobic conditions.
    i. For processing under anaerobic conditions, initiate anaerobic incubation for two plates and aerobic incubation for one plate at 36±1°C for 48±4 h. When processing outside of anaerobic conditions, initiate anaerobic incubation of two plates and aerobic incubation of 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 any growth on the plate incubated aerobically. Record observations.

2. 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.

3. 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.

4. Seal inoculated plates with Parafilm, or equivalent, to prevent dehydration during incubation in the anaerobic chamber. Invert plates and incubate anaerobically for 10 days at 36±l°C and approximately 70% relative humidity (applicable only for chamber).

5. 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. If growth is not confluent, discard all plates and re-initiate a new set. Record observations.

6. On day 10, discontinue incubation and transfer the CABA plates to a BSC. 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.

7. Degree of conversion of vegetative cells to spores should be approximately 90%. See Attachment 1 for example of conversion process over the incubation period.

8. 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.

9. Using a 10 mL sterile serological pipette, aspirate as much of the microbial suspension as possible from each plate, pool it in a sterile 50 mL plastic conical tube, and mix well by thorough vortexing. Divide the microbial suspension evenly into two 50 mL conical tubes. Mix well by thorough vortexing.

10. Centrifuge tubes at 4,500×g for 15 min.

11. 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.

12. 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.

13. 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.

14. 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 (make sure 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.

15. Following the heat treatment, allow the suspension to cool to room temperature. Mix the spore suspension 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%.

16. Mix the spore suspension vigorously by vortexing (30±5 s) prior to taking an aliquot of the spore suspension (e.g., 10 µL). Perform serial 10-fold dilutions of the spore suspension out to 10^{-7} in PBS-T.

17. Spread-plate 0.1 mL of the 10^{-6} and 10^{-7} dilutions on BHIY-HT in duplicate.

18. 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 $>10^{8}$ viable spores/mL. Store spore suspension at 2-8ºC for up to 5 days prior to purification.

E. Spore Purification

1. Prepare a 50% (w/v) solution of HistoDenz™ in sterile deionized water and pipet 5 mL into each of four sterile 15 mL plastic conical tubes. Bring the HistoDenz™ and spore preparation to room temperature before use.

2. Layer 1 mL of spore suspension on top of each of the four tubes of HistoDenz™. Centrifuge tubes at 4,500×g for 10 min using a swinging bucket rotor.

3. 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.

4. 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 cold (2-8°C) PBS-T.

5. 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 a siliconized microcentrifuge tube.

6. 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 cold PBS-T.

7. Cap the tubes and mix thoroughly by vortexing (30±5 s) to break up the pellets (ensure absence of visual clumps or fragments of the pellet).

8. Centrifuge the microcentrifuge tubes at 16,000×g for 2 min. Discard the supernatant and resuspend the pellets in 1 mL of cold 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.

9. Repeat washing procedure two additional times, for a total of three washes.

10. After the third wash, discard the supernatant and resuspend the pellets in each microcentrifuge tube with 0.5 mL of sterile cold PBS-T. Pool the contents of each microcentrifuge tube into one 15 mL conical tube. This is the purified spore suspension. Store the purified spore suspension at 2-8°C for up to 10 days prior to freezing.

11. Determine spore purity by inspecting under phase-contrast microscopy. Spores appear bright and ovular, while vegetative cells appear dark and rod-shaped. Calculate purity of the spore suspension using the formula presented in section V.1. The purity of spores should be ≥95%. See Attachment 2 for example of a purified spore suspension.

12. Determine titer of the purified spore suspension as in IV.d.16-18; use the formula presented in V.2 for calculations. The purified spore suspension should be approximately 10^9 viable spores/mL.

13. Based on the titer calculated in V.2, dilute a small aliquot (e.g., 100 µL) of the purified spore suspension with PBS-T to achieve a required concentration of approximately 5.0×10^8 viable spores/mL.

14. 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.

15. Once the titer of the aliquot is confirmed to be within the acceptable concentration, dilute the remainder of the purified spore preparation following the dilution scheme.

16. Determine the titer of the diluted purified spore suspension from step IV.E.15. Attain a titer of approximately 5.0×10^8 spores/mL.

17. For confirmatory purposes, conduct biochemical and antigenic analyses or other comparable confirmation procedures (e.g., Vitek).

F. Long Term Spore Storage

1. Freeze spores within 10 days of purification (step IV.E.10). Aliquot the diluted purified spore suspension into cryovials (~500 µL/vial) and store at -80±5°C. Store/use frozen spores for up to 90 days. Each cryovial is for single use only.

G. Qualifying Spores with Sodium Hypochlorite (NaOCl)

1. Within 2-10 days of storage, qualify the frozen spore suspension by conducting the OECD Quantitative Method (SOP MB-31) using two concentrations of laboratory grade NaOCl.

2. Using sterile deionized water as the diluent, prepare a 5,000±250 ppm and a 1,500±150 ppm solution of NaOCl.

3. Verify the concentration of the NaOCl solutions using an appropriate titration procedure (e.g., Hach digital titrator) prior to use. Use NaOCl solutions within 3 h of preparation.

4. Refer to sections IV.C and IV.D of SOP MB-31 for *Preparation of final spore suspension (with soil load)* and *Inoculation and drying of carriers*, respectively.

5. Inoculate a minimum of eleven carriers (e.g., three for each concentration of NaOCl, three for control, and two extras).

6. Use 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).

7. Control counts should be 6.0-7.0 logs/carrier.

8. 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}

9. The spore suspension is qualified if the following log reduction values are observed:

   i. 5,000 ppm: >5

   ii. 1,500 ppm: <3

H. Acceptance Criteria

1. The spore suspension is acceptable for use if all required criteria have been met:

   i. Spore titer of approximately 5.0×10^8 viable spores/mL.

   ii. Mean spore purity of ≥95%.

   iii. Required mean LR against two concentrations of NaOCl (>5 for 5,000 ppm and <3 for 1,500 ppm).

V. Data Analysis and Calculations

1. Determine spore suspension purity by the following formula:

$$\%\ Purity = 100\ \% \times \frac{A}{A+B}$$

   Where *A* = mean spore count and *B* = mean vegetative cell count.

2. Determine the titer of the spores in suspension using the following formula:

$$\text{Spores as CFU/mL} = \frac{\text{A} \times \text{B}}{\text{C}}$$

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

3. Per test, colony counts are recorded and used in calculations to determine log reductions.

4. To calculate the Colony Forming Units (CFU)/carrier (control or treated), use the following equation: $\left(\frac{CFU\ \ for\ 10^{-y} + CFU\ \ for\ 10^{-z}}{(a\times10^{-y}) + (b\times10^{-z})}\right)\times c$, where 10^{-y} and 10^{-z} are the dilutions filtered, "a" and "b" are the volumes filtered at each dilution (typically 9 or 10 mL), and "c" is the volume of medium originally in the vial with the carrier (10 mL). Account for the volume filtered in the calculations.

   i. *Example*. When 10^0 dilution yields 130 CFU and the 10^{-1} dilution yields 20 CFU:

$$\left(\frac{130\ CFU + 20\ CFU}{(9\ mL\ \times 10^0) + (10\ mL\ \times 10^{-1})}\right) \times 10\ mL\ (vol.in\ vial) = 1.5 \times 10^2\ CFU/carrier$$

5. When TNTC values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and account for the dilution factor in the calculations.

   i. *Example*. When 10^0 dilution yields TNTC CFU and the 10^{-1} dilution yields TNTC CFU:

$$\left(\frac{200\ CFU}{10\ mL \times 10^{-1}}\right) \times 10\ mL\ (vol.in\ vial) = 2.0 \times 10^3\ CFU/carrier$$

6. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).

7. Calculate the mean $\log_{10}$ density across treated carriers.

8. Calculate the mean $\log_{10}$ density across control carriers.

9. Calculate the $\log_{10}$ reduction (LR) for treated carriers: $\log_{10}$ reduction = mean $\log_{10}$ density for control carriers - mean $\log_{10}$ density for treated carriers.

10. For a set of 3 treated carriers: when the 10^0 dilution (the contents of the vial with the carrier) is filtered either by itself or in addition to other dilutions and the data for each carrier result in zeros for each dilution filtered, report the LR as greater than or equal to the mean $\log_{10}$ density for the control carriers.

VI. Attachments

A. Attachment 1: Photograph of percent sporulation of *C. difficile*

B. Attachment 2: Photograph of purified *C. difficile* spores

VII. References

A. ASTM E2839-11, Standard Test Method for Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents. ASTM International, West Conshohocken, PA, 2011.

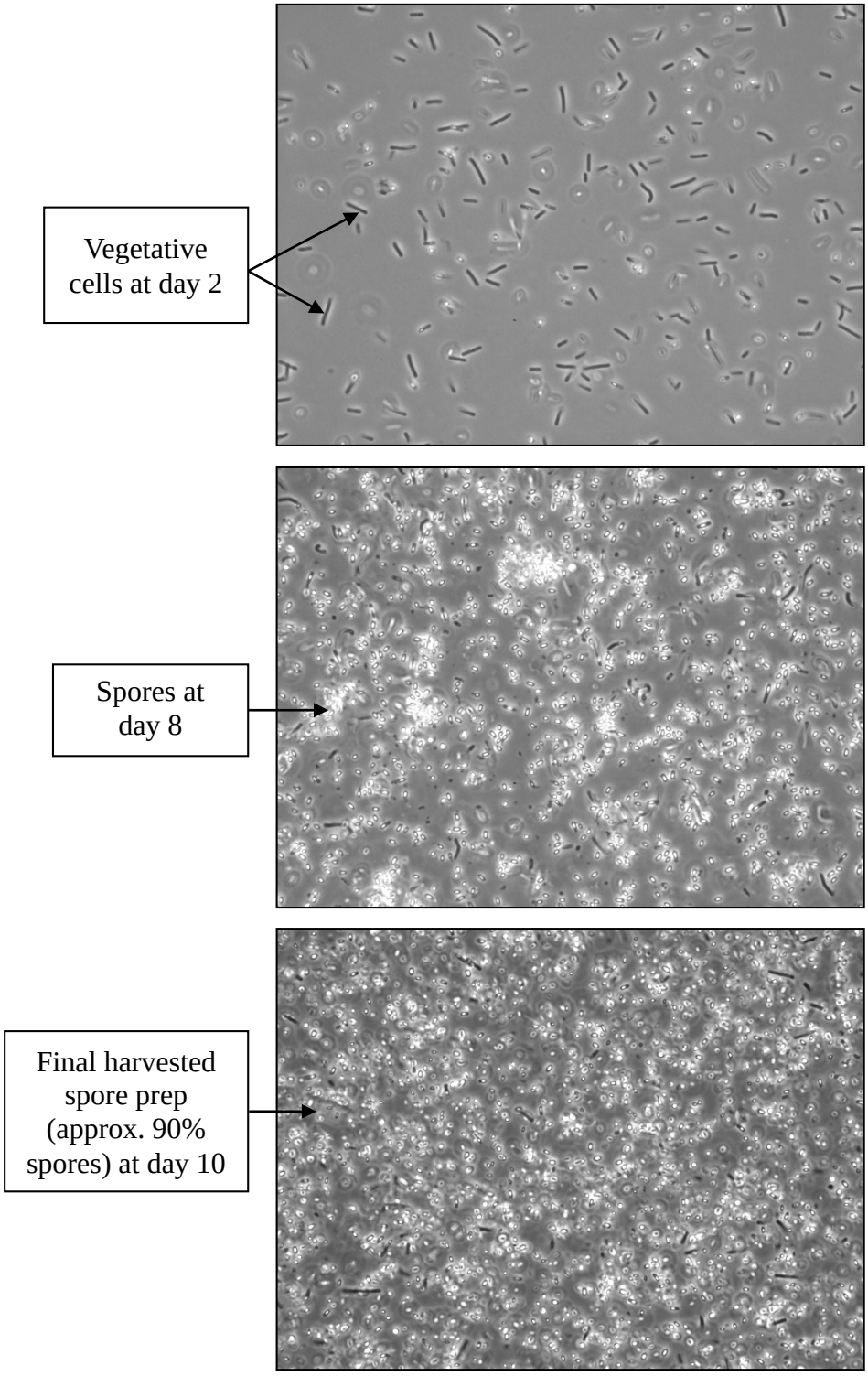
B. Hasan, J. A., Japal, K. M., Christensen, E. R. and Samalot-Freire, L. C., "Development of methodology to generate *Clostridium difficile* spores for use in the efficacy evaluation of disinfectants, a pre-collaborative investigation," *J. AOAC Int*, Vol 94, 2011, pp. 259-272.

C. Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington, D.C., 2012.

D. Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Ed., Centers for Disease Control and Prevention, and National Institute of Health, Washington D.C., 2009.

Attachment 1

Photograph of percent 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™, depicting ≥95% purity