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

SOP Number: MB-28-04

Date Revised: 06-19-14

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Title	Production of Spores of *Clostridium difficile* for Use in the Efficacy Evaluation of Antimicrobial Agents
Scope	Describes the test methodology, based on ASTM Standard E2839-11, for producing standardized spore suspensions of *C. difficile*.
Application	For use in the evaluation of antimicrobial products with *C. difficile* claims.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	1. Pre-reduced medium: Medium free of oxygen. 2. Density gradient medium: HistoDenz™ is a non-ionic gradient medium used to separate spores from vegetative cells and cell fragments on the basis of density. 3. Purified spores: Spore suspension exhibiting ≥95% spores following processing with Histodenz. 4. 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 Material 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-02 (Thermometers and hygrometers) and EQ 05 for details on method and frequency of calibration.
5. Sample Handling and Storage	Not applicable
6. Quality Control	Quality Control information is documented in the method and on the appropriate form(s) (see section 14).
7. Interferences	The test organism must be incubated under strict anaerobic conditions. If an anaerobic environment is not maintained, the elevated oxygen will compromise the viability of *C. difficile*.
8. Non-conforming Data	Procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived 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. RCM) is pre-reduced for at least 24±2 h prior to use. 3. Place inoculated plates under anaerobic conditions within 1 hour.
11. Special Apparatus and Materials	1. *Biosafety cabinet*—For maintaining an aseptic work environment. 2. *Sterile centrifuge tubes*—Polypropylene, 15 mL and 50 mL graduated plastic centrifuge tubes with conical bottoms.

3. *Centrifuge with swinging-bucket rotor*—To allow sedimentation of spores for washing and/or concentration.
4. *Micropipette*—Calibrated.
5. *Positive displacement pipette*—To dispense spores with 10 µL pipette tip.
6. *Timer*—Any certified timer that can display time in seconds.
7. *Test tubes*—Reusable or disposable 20 × 150 mm for cultures/subcultures.
8. *Inoculating loop*—10 µL transfer loop.
9. *COY Anaerobic chamber*—Supported by a gas mixture consisting of 10% Hydrogen, 5% CO_2, and 85% N_2.
10. *Anaerobic incubator*—Use the incubator at 36±1°C inside the COY anaerobic chamber to support the growth of the organism.
11. *Microscope* with 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option.
12. *Vortex mixer*.
13. *Serological pipettes*—Sterile single-use pipettes of 10.0, 5.0, and 1.0 mL capacity.
14. *Cell Scraper*—To gently scrape plates to remove spores for harvesting.
15. *Plate spreader*—To spread inocula on agar to create a uniform lawn.
16. *Microcentrifuge tubes*—Sterile 1.5-mL low-retention (siliconized) microcentrifuge tubes. Use for dilutions, processing of spores during purification and HCl resistance testing.
17. *Cryovials*—Sterile 2.0 mL cryovials.
18. *Parafilm™*—to seal plates.
19. Media and Reagents
    a. *Reinforced clostridial medium (RCM)*—For use in rehydrating lyophilized/frozen vegetative culture of test organism. Prepare RCM according to manufacturer's instructions, and pre-reduce in an anaerobic environment for 24±2 h prior to use.
    b. *RCM plus 15% glycerol (Cryoprotectant)*—For use as maintenance and cryopreservation medium for vegetative frozen stock (VFS) cultures. Prepare RCM and add 15% glycerol, autoclave for 20 min at 121°C, and pre-reduce in an anaerobic environment for 24±2 h prior to use.
    c. *CDC anaerobic 5% sheep blood agar (CABA)* plates—Commercially available and pre-reduced, used for sporulation.

	d. *Brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT)*—Commercially available and pre-reduced, used as recovery medium for enumeration of viable spores. e. *Phosphate-buffered saline (PBS)*—Prepare 10X stock solution of PBS by dissolving entire contents of bottle of Fisher Bioreagents BP661-10 Phosphate Buffered Saline Powder Concentrate in 1 L of deionized water. Dilute 1:10 (1 part 10X solution plus 9 parts deionized water) to obtain 1X solution, distribute into bottles and sterilize appropriately. f. *Tween-80 (polysorbate 80)*—To prepare Phosphate-buffered saline (PBS) containing 0.1% Tween 80. g. *Phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 80 (PBS-T)*—Diluting and washing reagent; add 2.0 mL of polysorbate 80 (Tween 80) to 200 mL PBS (10X) solution. Mix thoroughly and (using a volumetric flask) bring solution to volume (2 L) with deionized water. Distribute into bottles and filter sterilize. h. *Water*—Use sterile deionized water as diluent. i. *Certified Hydrochloric acid*—Use 2.5 M HCl for quantitative acid resistance test. j. *HistoDenz™*—Prepare a 50% (w/v) solution in deionized water. This is a density gradient medium for spore purification. Pass the solution through a sterile 0.45 μm filter to sterilize. 20. Test Organism a. *Clostridium difficile* (ATCC 43598), a toxigenic strain (*tcdA*-, *tcdB*+), obtained from ATCC or another reputable vendor. 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 CABA medium within 48 h at 36±1ºC.
12. Procedure and Analysis	
12.1 Preparation of Frozen Stock Cultures of Test Organism	a. *Clostridium difficile received in lyophilized vegetative form*: To reinitiate a new stock culture, reconstitute contents of the lyophilized culture with 0.5 mL of sterile pre-reduced RCM in the COY anaerobic chamber as per the manufacturer's instructions. After rehydration, aseptically transfer the vial contents to a tube containing 4±1 mL of pre-reduced RCM, and mix by gentle vortexing.

	b. *Clostridium difficile received as frozen vegetative culture*: To reinitiate 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 COY anaerobic chamber, and mix by gentle vortexing. c. Spread plate 100 µL of the reconstituted culture on five CABA plates. Also streak one CABA plate for isolation to check for culture purity. Invert plates and incubate anaerobically at 36±1°C for 48±4 h. d. Following incubation, add 2 mL of RCM plus 15% (v/v) glycerol to each CABA plate. e. Using a sterile cell scraper, gently scrape culture from the surface of the plate, aspirate with a pipette and transfer to a 15-mL conical tube. f. Repeat this process for the remaining four plates. Pool the suspensions, mix thoroughly, and pipette 0.5mL aliquots into cryovials; cap tightly. g. Store the cryovials at −80±5°C for 18 months. These tubes are the Vegetative Frozen Stock (VFS) Culture. h. After a minimum of one week of freezing, thaw a VFS cryovial at room temperature inside the COY chamber. i. Vortex suspension thoroughly, and dilute 0.1 mL in a 1:10 series out to 10^{-6} in PBS-T. Spread-plate 100 µL of diluted suspension on BHIY-HT in duplicate. Invert plates and incubate anaerobically at 36±1°C for 48±4 h. j. Record the number of CFU/plate to determine the CFU/mL. The titer should be $>1.0 \times 10^8$ CFU/mL.
12.2 Preparation of a spore suspension from VFS	a. Streak three CABA plates with the VFS. Incubate two plates anaerobically, and the third one aerobically at 36±1°C for 48±4 h. 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 growth on the plate incubated aerobically. b. Using one of the two plates (incubated anaerobically) from 12.2a, 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. After incubation, inoculate ten CABA plates each with 100 µL of the RCM broth culture. Spread the inoculum evenly using a disposable sterile spreader to create a lawn. *Note*: For every 10 CABA plates inoculated, the resulting pellet is resuspended in 4 mL of PBS-T (see 12.2 m below). Follow the ratio

	(4 mL per 10 plates) if additional plates are harvested. d. 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. Based on experience, optimal sporulation is reached at day 10. e. Inspect one plate after 24±2 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 inoculate a new set of ten plates. f. On day 10, discontinue incubation and place the CABA plates inside a BSC. Prepare wet-mount samples of *C. difficile* from a sample plate and inspect under phase-contrast microscopy. Spores appear bright and ovular, while vegetative cells appear dark and rod-shaped. g. Degree of conversion of vegetative cells to spores should be approximately 90%. See Attachment 1 for example of conversion process over the incubation period. h. Harvest growth from each plate by adding 5 mL of PBS-T to each plate, and 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. i. Using a 10 mL sterile serological pipette, aspirate as much of the microbial suspension as possible from each plate, and pool it in a sterile 50 mL plastic conical tube. Divide evenly into two 50 mL conical tubes. Mix well by through vortexing. j. Centrifuge tubes at 4500 × g for 15 min. k. Discard the supernatant and resuspend the pellets with 30 mL of PBS-T. Cap the tubes tightly and disaggregate the pellet by vortexing. This step is the first wash. l. Repeat the washing step. After the second wash, disaggregate the pellets from each tube, and combine into one tube for the third wash. Mix well by vortexing. Centrifuge tubes at 4500 × g for 15 min. m. 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. n. 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. o. Following the heat treatment, allow the suspension to cool to room

	temperature. p. Prepare a wet-mount of the well-vortexed, heat-treated spore suspension and observe at least five fields using a phase-contrast microscope. The percent of spores to vegetative cells should be approximately 90% (or higher). q. Mix the spore suspension vigorously by vortex prior to taking an aliquot of the spore suspension (e.g., 10 µL). Perform serial 10-fold dilutions of the spore suspension (e.g., out to 10^{-7}) in PBS-T. r. Spread-plate 0.1 mL of the appropriate dilutions on BHIY-HT in duplicate. s. Store remaining spore suspension at 4ºC for up to 5 days prior to purification. t. Once the inocula have dried, invert plates and incubate anaerobically at 36±1°C for 24±4 h. Record the number of colony forming units (CFU). The titer must be $>10^8$ viable spores/mL.
12.3 Spore Purification	a. Make 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. The number of 15 mL plastic conical tubes is proportional to the reconstitution volume, see step 12.2 m above. If HistoDenz and spore preparation are refrigerated, bring to room temperature before use. b. Layer 1 mL of spore suspension on top of each of the four tubes of HistoDenz. c. Centrifuge tubes at 4,500×g for 10 min using a swinging bucket rotor. d. 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. e. Using a repetitive pipetting action, resuspend and mix the pellet (without touching the pellet) with a 1 mL micro pipette. Bring the volume up to approximately 1 mL with cold PBS-T. f. Mix throughly by vortex 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 micro-centrifuge tube. g. Centrifuge the microcentrifuge tubes (four total tubes) at 16,000×g for 5 min. Discard the supernatant and resuspend the pellet in 1mL of cold (2-5°C) PBS-T. h. Cap the tubes and mix throughly by vortex to break up the pellet

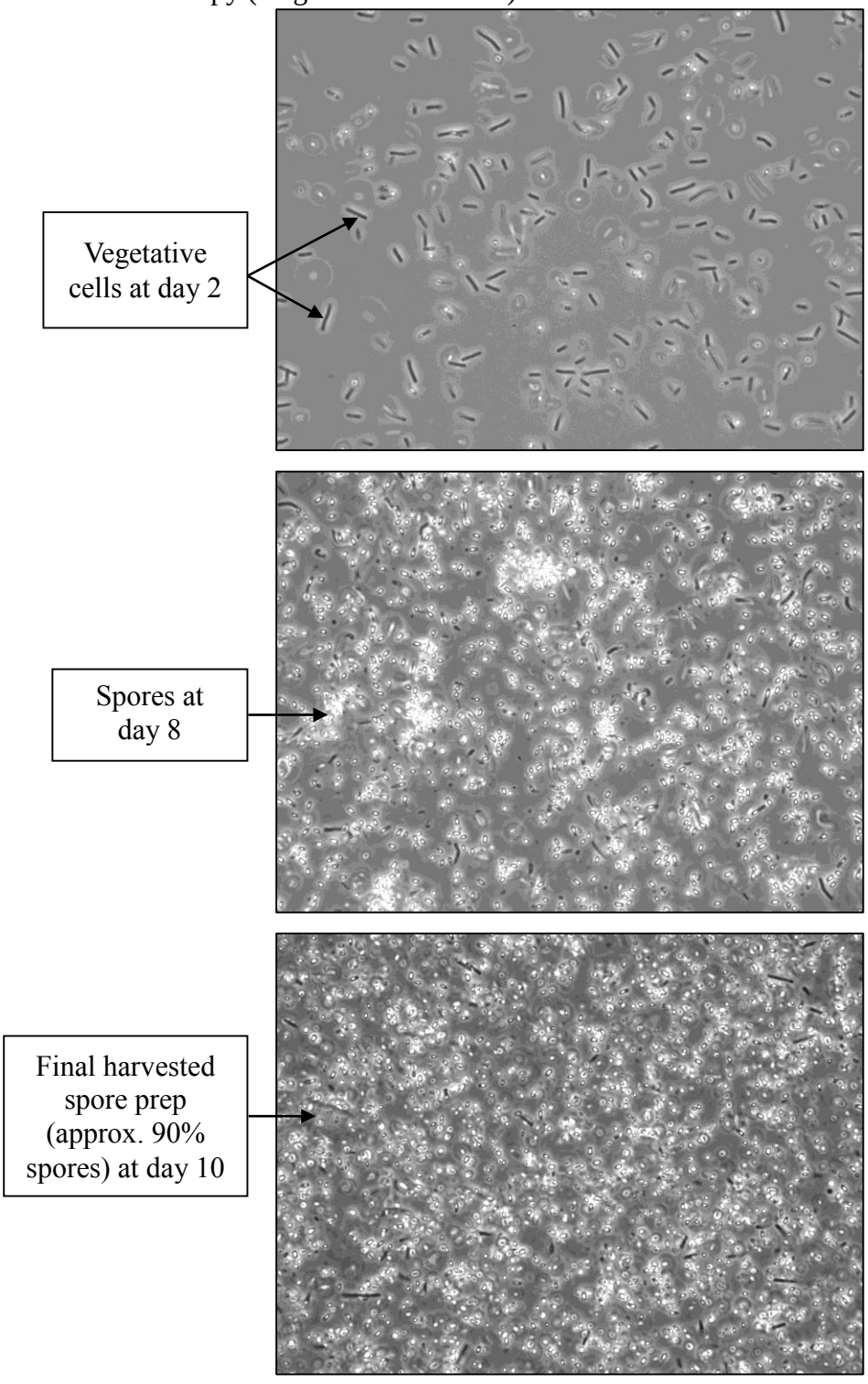
	(ensure absence of visual clumps or fragments of the pellet). i. Centrifuge the microcentrifuge tubes at 16,000 × g for 2 min. Discard the supernatant and resuspend the pellet in 1 mL of cold PBS-T. Cap the tubes and mix throughly by vortex to break up the pellet (ensure absence of visual clumps or fragments of the pellet). This step is the first wash. j. Repeat washing procedure two additional times, for a total of three washes. k. After the third wash, discard the supernatant and resuspend the pellet in each microcentrifuge tube with 0.5 mL of sterile PBS-T and pool (e.g., pool into one 15 mL sterile conical tube). This is the final purified spore suspension. l. Determine spore purity using procedures stated in microscopic evaluation of spore suspension (see 12.2 p). Calculate purity of the spore suspension using the formula presented below in 13.1. The purity of spores should be ≥95%. See Attachment 2 for example of a purified spore suspension. m. Determine titer of the purified spore suspension as in 12.2 (q-t); use the formula presented in 13.2 for calculations. Repeat titer assessment at least one more time (i.e., two separate titer evaluations). The average titer of purified spore suspension from two determinations should be $\geq 10^9$ viable spores/mL. n. Based on the titer calculated in 12.3 m, dilute a small aliquot (e.g., 100 µL) of the purified spore suspension to achieve a desired concentration of approximately 5.0×10^8 viable spores/mL with PBS-T. o. Determine titer of spores from the diluted aliquot by making serial dilutions of the aliquot and plating 0.1 mL on BHIY-HT plates. Incubate plates anaerobically for 24±2 h at 36±1°C. Record the number of CFUs and calculate titer. p. Once the titer is confirmed to be within the acceptable range, dilute the remainder of the purified spore preparation to achieve the spores/mL as noted in 12.3 n. This is the final spore suspension. q. Determine the titer of the final spore suspension. The titer must be between $2.0 \times 10^8 – 8.0 \times 10^8$ spores/mL. Once determined, proceed to HCl resistance test and aliquot for long term storage. Note: To allow for titer assessment and acid resistance testing, the purified spore suspension may be stored at 4ºC for up to 10 days.
12.4 Quantitative Hydrochloric	a. Perform HCl resistance on the final spore suspension. Place 990 µL of 2.5 M HCl into one siliconized microcentrifuge tube (treatment

Acid (HCl) Resistance Test	tube) and 990 µL of PBS-T into one siliconized microcentrifuge tube for the control. b. Mix the final spore suspension vigorously by vortex. In a timed sequence and using a positive displacement pipette, transfer10 µL of well-mixed spore suspension into the microcentrifuge treatment and control tubes and briefly vortex each tube. c. Allow the tubes to remain at room temperature for 10 min±30 sec. d. Following the 10 min exposure time, transfer 0.1 mL from each tube (in sequential order) to tubes containing 900 µL of PBS-T (i.e., the neutralizer). e. Serially dilute the suspensions (e.g., out to 10^{-6}) in PBS-T and spread-plate 0.1 mL aliquots from appropriate dilutions, in duplicate, on BHIY-HT. Invert plates and incubate for 48±4 h at 36±1°C under anaerobic conditions. f. Determine the log reduction following the HCl treatment using the formula presented in 13.3. g. The spores are considered acid resistant if the log reduction (control minus treated) is between 0 and 2.
12.5 Long Term Spore Storage	a. The spore suspension is considered acceptable for use if all required criteria have been met: 1) spore titer of 2.0×10^8 to 8.0×10^8 viable spores/mL, 2) spore purity of ≥ 95%, and 3) acid resistance (log reduction between 0 to 2 following 10 min of exposure to 2.5 M HCl). b. Assign a preparation number to the final spore suspension. c. Make aliquots in cryovials and label appropriately. To standardize long term storage conditions, store spore suspension in cryovials at -20ºC for up to 1 year.
13. Data Analysis/ 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. Determine the $\log_{10}$ reduction following HCl treatment:

	Log_{10} Reduction (LR) = LC-LH Where LC = Log_{10} of viable spores after control treatment, and LH = Log_{10} of viable spores after HCl treatment.
14. Forms and Data Sheets	1. Attachment 1: Monitoring percent sporulation of *C. difficile.* 2. Attachment 2: Purified *C. difficile* spores, using a density gradient medium (HistoDenz), depicting ≥99% purity. 3. Test Sheets: Test sheets are stored separately from the SOP under the following file names: *C. difficile* Spore Titer Form — MB-28-04_F1.docx HCl Resistance Test Form — MB-28-04_F2.docx HCl Resistance Test Dilution and Results Form — MB-28-04_F3.docx
15. References	1. 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. 2. EPA Guidance for the Efficacy Evaluation of Products with Sporicidal Claims against *Clostridium difficile*, http://www.epa.gov/oppad001/ cdif-guidance.html, 2009. 3. 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. 4. Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington, D.C., 2012. 5. 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

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



Attachment 2

Purified *C. difficile* spores (ATCC 43598), using HistoDenz, depicting ≥99% purity