US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
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Standard Operating Procedure for Quantitative Disk Carrier Test Method (QCT-2) Modified Modified for Testing Antimicrobial Products Against Spores of *Clostridium difficile* (ATCC 43598) on Inanimate, Hard, Non-porous Surfaces

SOP Number: MB-31-03

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Title	Quantitative Disk Carrier Test Method (QCT-2) Modified for Testing Antimicrobial Products Against Spores of *Clostridium difficile* (ATCC 43598) on Inanimate, Hard, Non-porous Surfaces.
Scope	This quantitative method is used to evaluate the sporicidal efficacy of liquid disinfectants against spores of *Clostridium difficile* (ATCC 43598) on inanimate, hard, non-porous surfaces. This SOP is based on the ASTM Standard E2197-11(see 15.1) and incorporates methodologies specific to testing spores of *C. difficile*.
Application	Data from this method are used to generate the log reduction (LR) values of viable spores of *C. difficile* as the quantitative measure of efficacy for liquid disinfectants on an inanimate, hard non-porous surface.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer:	_____ Print Name: _____
Quality Assurance Unit:	_____ Print Name: _____
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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Eluate = recovered eluent that contains the test organism. 2. Eluent = the liquid that is added to a vial containing the carrier to recover the test organism. 3. Frozen spore suspension = frozen stocks of spore suspension prepared per SOP MB-28. 4. Final test suspension = thawed frozen spore suspension including the addition of the soil load when specified.
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 the test substance.
3. Personnel Qualifications and Training	1. A reference standard (e.g., predetermined concentrations of sodium hypochlorite) may be used to check method performance and analyst proficiency. 2. 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 (Balances), EQ-05 (Timers) and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22; Disinfectant Sample Preparation, and SOP COC-01; Disinfectant Sample Login, Tracking and Disposal.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Prior to efficacy testing, verify neutralizer effectiveness using the procedure outlined in SOP MB-26 (Neutralization of Microbicidal Activity using the OECD Quantitative Method) with the following modification: For Titer Control use PBS with 0.1% (v/v) Tween-80 (PBS-T) instead of PBS. 2. The test organism (*C. difficile* ATCC 43598) must be incubated under strict anaerobic conditions. The presence of oxygen will severely compromise the viability and growth of *C. difficile.* 3. During testing, do not process a carrier where the test substance runs off the carrier; replace with an untreated inoculated carrier and vial.
8. Non-conforming Data	1. The range for the control carrier counts is $>10^6$ to $<10^7$ spores/carrier.

9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.
11. Special Apparatus and Materials	1. *Test microbe*. Spore suspension of *C. difficile* (ATCC 43598); prepared according to MB-28. 2. *Recovery medium*. Brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT). Pre-reduced recovery medium (Anaerobe Systems, Morgan Hill, CA) for enumeration of viable spores. 3. Reagents a. *Neutralizer in eluent.* The default neutralizer is PBS with 0.1% Tween-80; however, an alternative neutralizer may be used. b. *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. c. *Tween-80 (polysorbate 80).* To prepare Phosphate-buffered saline (PBS) containing 0.1% Tween 80. d. *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. e. *Spore stain*. 5% aqueous malachite green and 0.5% aqueous safranine to differentiate spores from vegetative cells. Spores appear green while vegetative cells appear red. f. *Soil load*. The recommended soil load (if required) to be incorporated in the test suspension is a mixture of the following stock solutions in PBS: i. BSA: Add 0.5 g bovine serum albumin (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 minutes at 121°C), aliquot and store at -20 ± 5°C.

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

g. *Test substance.* Refer to SOP MB-22, Disinfectant Sample Preparation.

h. *Test substance diluent.* The test substance diluent will be used as specified. For preparation of hard water, refer to SOP MB-30.

i. *Water.* Either de-ionized distilled water or water with equivalent quality for making reagent solutions and culture media.

j. *Liquinox.* To clean carriers.

4. Apparatus

a. *Calibrated positive displacement pipette (10 µL).* For carrier inoculation use a 10 µL positive displacement pipette tip.

b. *Micropipette* (200 µL). For deposition of 50 µL test substance on inoculated carrier.

c. *Carriers*. Disks (1 cm in diameter) made from 0.7 mm thick sheets of brushed and magnetized stainless steel (AISI #430). 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 Attachment 1 for complete specifications.

d. Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes used in rinsing of vials and filters.

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

f. *Magnet.* Strong enough to hold a treated carrier in place in the vial while the liquid is being poured out of it for membrane filtration.

g. *Polyethersulfone membrane filter (PES).* For recovery of test microbe, 47 mm diameter and 0.2 µm pore size. Filtration units (reusable or disposable) may be used.

	h. *Sterile vials (plastic or comparable)*. To hold test carriers: flat bottom and wide-mouth (at least 25mm in neck diameter) to accommodate addition and removal of the carriers, for holding inoculated carriers to be exposed to the test substance and for accommodating neutralizer/eluent. i. *Vortex mixer*. To vortex the eluate and rinsing fluid in the carrier vials to ensure efficient recovery of the test organism(s). j. *Certified timer*. That can be read in minutes and seconds for the contact time specified. k. *Desiccator with desiccant (e.g., $CaCO_3$)*. For drying the inoculum on the carriers. l. *Vacuum source*. In-house line or suitable vacuum pump (20-25 in. mercury) for drying carriers and for filtering. m. *Petri dish (100 × 15mm)*. To hold carriers. n. *Filter paper*. Whatman No. 2, to line Petri dishes. o. *COY Anaerobic chamber*. Supported by a compressed gas mixture consisting of 10% Hydrogen, 5% CO_2, and 85% N_2. To provide an anaerobic environment. p. *Anaerobic incubator*. Use the incubator at 36 ± 1°C inside the COY anaerobic chamber to support the growth of *C. difficile*. q. *Microscope*. With 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option. To examine spores.
12. Procedure and Analysis	Conduct three independent tests (three test days) or as specified by the study sponsor. The product performance standard for a *C. difficile* claim is a minimum 6 log reduction in viable spores for each test. For each test, evaluate 10 treated and 3 control carriers.
12.1 Preparation and sterilization of carriers	a. Visually check the brushed top surface of the carriers (with the rounded edge) for abnormalities (e.g., rust, chipping, deep striations) and discard if observed. Record physical screening of carriers on form as noted in section 14. b. Soak visually screened carriers in a suitable detergent solution (e.g., 1% v/v Liquinox) for 1-2 h to degrease and then rinse thoroughly in distilled water. c. Place up to 20 clean dry carriers on a piece of filter paper inside the bottom surface of a glass Petri dish (150 mm in diameter). Cover the Petri dish with lid and sterilize. After sterilization, aseptically

		transfer carriers to sterile Petri dishes without filter paper for inoculation.
12.2 Preparation of test organism	a.	Preparation of Frozen Stock of Spore Suspensions. Refer to SOP MB-28 for the generation of purified spores of *C. difficile* (ATCC 43598).
12.3 Preparation of the final test suspension with soil load	a.	Defrost a cryovial of *C. difficile* spores (approximately 10-15 min at room temperature). Each cryovial is single use only.
	b.	Vortex the thawed spore suspension until re-suspended to evenly distribute the spores.
	c.	If soil load is required, to obtain 500 µL of the final test suspension vortex each component and combine the following:
		i. 25 µL BSA stock
		ii. 35 µL yeast extract stock
		iii. 100 µL mucin stock
		iv. 340 µL spore suspension
	d.	If different total volumes of the final test suspension are required, scale each component listed in 12.3c, maintaining the correct ratio of each component. Following the addition of the soil load, vortex mix the final test suspension.
12.4 Inoculation and drying of carriers	a.	Inoculate a minimum of fifteen carriers (e.g., ten for treated carriers, three for control carriers, and two extras). For inoculation, withdraw 10 µL of the final spore suspension with a calibrated positive-displacement pipette with a 10 µL pipette tip and deposit spore suspension in the center of each carrier.
	b.	Avoid contact with carrier and do not spread the spore suspension with the pipette tip. Use the same pipette tip to inoculate each batch of carriers. Discard any inoculated carrier where the final spore suspension has run over the edge.
	c.	Dry the carriers inside a Petri dish (with the lid off) in the BSC for 30±5 min. After the inoculum has dried, place the Petri dish in a desiccator connected to a vacuum line. Remove the Petri dish lid. Cover the desiccator and make sure that it is properly sealed. Continue drying under vacuum for 2 h at room temperature inside the BSC.
	d.	At the end of the drying period, turn off the vacuum, and cover the plate. Observe the dried inoculum on each carrier. Discard any carrier in which the inoculum has run off the surface. The carriers are

	stored overnight in the desiccator without vacuum for use the next day. Use dried carriers within 24 h of inoculation.
12.5 Exposure of the dried inoculum to the test substance or PBS-T (control counts)	a. Using sterile forceps transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred. b. Use a certified timer to ensure that each carrier receives the required exposure time (e.g., 5 min ± 3 sec). c. In a timed fashion, deposit 50 µL of the test substance, equilibrated to 20-25°C, over the dried inoculum on each test carrier, ensuring complete coverage, at predetermined staggered intervals. Use a new tip for each carrier; do not touch the pipette tip to the carrier surface. Do not cap the vials. d. Hold the test carriers at 20-25°C for the contact period. e. Treat control carriers last – each control carrier receives 50 µL of PBS-T, equilibrated to 20-25°C, instead of the test substance. Hold the control carriers at 20-25°C for the contact period.
12.6 Neutralization of test substance and elution of test organisms	The neutralizer for the control carriers is the same as that for the treated carriers. a. Within ± 3 seconds of the end of the contact period, add 10 mL of neutralizer at room temperature to each vial in the specified order, including controls, according to the predetermined schedule. The neutralized vial is the 10^0 dilution. Cap the vial and briefly (2-3 sec) vortex following the addition of the neutralizer. b. Following the neutralization of the entire set of carriers, vortex each vial for 30 ± 5 sec at high speed to recover the inoculum; ensure that the carrier is vortexing along with the liquid in the vial. Visually examine each carrier and, in case of incomplete elution, perform further vortexing to remove inoculum. Do not remove the carrier from the vial.
12.7 Dilution and recovery	a. Initiate dilutions within 30 min at room temperature after neutralization. Initiate filtration within 30 min of preparing the dilutions. Use 0.2 µm PES membrane filters. Direct plating is not allowed. b. Process the treated carriers first. Prior to filtering the contents of tubes and vials, pre-wet each membrane filter with approximately 10 mL of sterile PBS. c. For treated carriers, filter the entire contents of the vial (10^0) unless

	the study protocol calls for preparing serial dilutions. If dilutions are necessary, briefly vortex the vial and transfer 1 mL of the mixture into a 9 mL tube of PBS-T; proceed with preparing serial dilutions in PBS-T. d. For filtering the treatment vial, briefly vortex contents and while holding a magnet to the bottom of the vial (to keep the carrier in place) pour the contents into a filter unit. Rinse the vial with ~20 mL of PBS, briefly vortex, and while keeping the magnet in place, pour the wash liquid into the same filter unit. Repeat this step three more times. Swirl the contents of the filter unit and apply the vacuum. With the vacuum on, rinse the inside surface of each filter unit with an additional ~40 mL PBS. e. For filtration of contents in tubes, briefly vortex and pour into filter. Rinse each tube once with ~10 mL of PBS, briefly vortex, and pour the contents of the tube into the same filter unit. With the vacuum on, rinse the inside surface of each filter unit with an additional ~20 mL PBS. f. For control carriers, prepare serial dilutions using 1 mL from the vial (10^0) in 9 mL PBS-T out to the 10^{-5} dilution to provide countable filters (up to 200 CFU/filter). Filter the contents of the 10^{-4} and 10^{-5} tubes. For filtration of contents of tubes, briefly vortex and pour into filter. Rinse each tube once with ~10 mL of PBS, briefly vortex, and pour the contents of the tube into the same filter unit. With the vacuum on, rinse the inside surface of each control filter unit with an additional ~20 mL PBS. g. Aseptically remove the membrane filter (treated samples first, followed by the controls) and place on the recovery medium (pre-reduced BHIY-HT). Open each sealed package inside the BSC just prior to placement of the membrane filter. Avoid trapping any air bubbles between the membrane filter and the agar surface. h. Place BHIY-HT plates with membrane filters under anaerobic conditions within 50±10 min of opening the package of plates. Incubate BHIY-HT plates with membrane filters under anaerobic conditions at 36±1°C.
12.8 Recording results	a. For control carriers, record results as colony forming units (CFUs) per filter at 48±4 h of incubation. For treated carriers, record results at 72±4 h of incubation. If no or few colonies are observed after 72±4 h of incubation, continue to incubate for an additional 48±4 h. Colony counts in excess of 200 CFUs per membrane filter should be recorded as Too Numerous to Count (TNTC). If no colonies are

present, record as zero.

b. Inspect the growth from one of the membrane filters for purity and typical characteristics of the test microbe (see Table 1). Observe growth from a typical colony on a membrane filter using spore staining or under phase contrast microscopy. Record results on the Test Microbe Confirmation Sheet.

Table 1. Characteristics of *C. difficile* (ATCC 43598)

Typical Diagnostic Characteristics	
BHIY-HT plate	Growth circular, entire edge, convex, smooth and grey colonies.*
Phase-contrast microscopy	Spores appear bright and ovular while vegetative cells appear dark and rod-shaped.
Spore staining	Spores appear green while vegetative cells appear red.

*At 48±4 h

13. Data Analysis/ Calculations

1. Colony counts (CFUs) at each dilution are recorded and used to calculate the log reduction in viable spores.
2. To calculate the CFU/carrier when two (2) serial dilutions are filtered, use the following example formula:

$$\left(\frac{CFU \ \ for\, 10^{-y} + CFU \ \ for\, 10^{-z}}{(a \times 10^{-y}) + (b \times 10^{-z})} \right) \times c$$

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 (10 mL) of neutralizer originally in the vial with the carrier.

   a. When TNTC values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and scale up accordingly for the calculations.

   b. When zeroes are observed for each dilution filtered, substitute 0.5 for the zero at the lowest (least dilute) dilution and scale up accordingly for the calculations.

3. Calculate the $\log_{10}$ density (LD) of each carrier by taking the $\log_{10}$ of the CFU/carrier.
4. Calculate the mean $\log_{10}$ density across treated carriers.
5. Calculate the mean $\log_{10}$ density across control carriers.
6. Calculate the $\log_{10}$ reduction (LR) for treated carriers:

	log_{10} reduction = mean log_{10} control – mean log_{10} treated 7. If no spores are recovered for any treated carrier, report the LR as greater than or equal to the mean log_{10} density for the control carriers.
14. Forms and Data Sheets	1. Attachment 1: Carrier Specifications 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record Form — MB-31-03_F1.docx QCT-2 Method for Sporicidal Activity: Organism Culture Tracking Form — MB-31-03_F2.docx QCT-2 Method for Sporicidal Activity: Test Microbe Confirmation Sheet (Quality Control) — MB-31-03_F3.docx QCT-2 Method for Sporicidal Activity: Test Information Sheet — MB-31-03_F4.docx QCT-2 Method for Sporicidal Activity: Time Recording Sheet — MB-31-03_F5.docx QCT-2 Method for Sporicidal Activity: Serial Dilution Plating/Tracking Form — MB-31-03_F6.docx QCT-2 Method for Sporicidal Activity: Results Sheet — MB-31-03_F7.docx QCT-2 Method for Sporicidal Activity: Test Microbe Confirmation Sheet — MB-31-03_F8.docx
15. References	1. ASTM Standard E2197-11. Standard Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Chemicals. ASTM International, West Conshohocken, PA, 2011.

Attachment 1

Carrier Specifications

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.27559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.