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Office of Pesticide Programs

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

Quantitative Method for Testing Antimicrobial Agents Against Spores of *C. difficile* on Hard, Non-porous Surfaces

SOP Number: MB-31-07

Date Revised: 09-22-22

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Revisions Made	• Minor editorial changes for clarification purposes. • Added language in carriers section; the top of the carrier is brushed; only the top is visually screened and inoculated (Section 11.4.a). • When preparing mucin, it is now passed through a 0.2 µm pore diameter membrane filter instead of autoclaved (Section 11.2.1.iii). • Footnotes changed from referencing ASTM E3218-19 to ASTM E3218-21. • Removed Attachment 3: Gravimetric and Physical Wetness Determination for Towelettes from SOP and put it into the guidance. • Updated ASTM International Standard reference from 2019 to 2021 and updated version number from E3218-19 to E3218-21.

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Title	Quantitative Method for Testing Antimicrobial Agents Against Spores of *C. difficile* on Hard, Non-porous Surfaces
Scope	This document describes a standardized approach to quantitatively determine the effectiveness of antimicrobial chemicals in treating hard, non-porous surfaces contaminated with spores of *Clostridioides difficile* (ATCC 43598) based on ASTM E3218-21.
Application	The test method was designed to determine the $\log_{10}$ reduction (LR) in spores on a hard, non-porous surface after exposure to a test chemical in a closed system.

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Frozen spore suspension = a preparation of bacterial endospores that is maintained at -80±5°C and have been prepared and qualified in accordance with SOP MB-28. Spore suspensions of *C. difficile* used in this test method may be stored for up to 90 days under the conditions provided in the definition. 2. Final test suspension = thawed frozen spore suspension including the addition of a soil load. 3. Test system control = a solution of 1,500±150 ppm laboratory grade sodium hypochlorite (NaOCl) used to validate each efficacy test.
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 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 (weigh 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; Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01; Chain of Custody.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Verify the neutralizer specified for the disinfectant test chemical in advance of efficacy testing. See Attachment 2. 2. *Clostridioides difficile* (ATCC 43598), formerly known as *Clostridium difficile*, is an obligate anaerobe and must be incubated under strict anaerobic conditions. *C. difficile* will not grow in the presence of oxygen. Verification of anaerobic conditions is required. 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. For an acceptable test, the $\log_{10}$ density (LD) for each control carrier should be 6.0-7.0 spores/carrier.

	2. The 1,500 ppm NaOCl test system control should exhibit an LR of <3.0 in spores using a contact period of 3 min±3 s.
9. Data Management	Archive the data 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*. *C. difficile* (ATCC 43598); spores prepared according to SOP MB-28. 2. *Recovery medium*. Brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT). For enumeration of spores, commercially available as pre-reduced (Anaerobe Systems, Morgan Hill, CA, or equivalent). 3. Reagents a. *Water.* Either de-ionized water or water with equivalent quality for making reagent solutions and culture media. b. *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; adjust pH to 7.0±0.5 if necessary. c. *PBS containing 0.1% (v/v) Tween 80 (PBS-T)*. Diluting reagent; adjust pH to 7.2±0.2 if necessary. d. *PBS-T with 0.1% (w/v) sodium thiosulfate*. Neutralizer for the test system control (1,500±150 ppm NaOCl); adjust pH to 7.2±0.2 if necessary. i. Confirm the effectiveness of PBS-T with 0.1% (w/v) sodium thiosulfate as a neutralizer for 1,500±150 ppm NaOCl using the procedure in Attachment 2. e. *Neutralizer.* Specific to disinfectant test chemical being evaluated as determined for effectiveness and toxicity according to Attachment 2. Use a neutralizer containing 0.1% (v/v) Tween-80 to reduce spore clumping. f. *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 (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, aliquot, and store frozen at -20±2°C.

iv. Aseptically aliquot soil stock solutions and store up to one year at -20±5°C. The stock solutions of the soil load are single use only; do not refreeze once thawed.
Note: intermittently vortex soil stock solutions while preparing aliquots. Other volumes of the stock solutions may be prepared following the same ratio.

g. *Test chemical.* Antimicrobial test solution.

h. *Reagent grade sodium hypochlorite* (NaOCl) *with total chlorine* ≥4%. To prepare 1,500±150 ppm total chlorine for test system control.

i. *Tween-80* (polysorbate 80). To make dilution blanks and neutralizers.

j. *Laboratory detergent (1% solution).* To clean carriers.

4. Apparatus

a. *Carriers:* Discs (1 cm in diameter) made of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side. The top of the carrier is brushed; only the top is visually screened and inoculated. Carriers are single-use only. See Attachment 1 for complete carrier specifications and photographs of screened carriers.

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

c. *Calibrated micropipettes* (e.g., 200 µL) with 10-100 or 20-200 µL tips. For preparing aliquots of soil and spores; for deposition of test chemical on carrier.

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

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

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

	g. *Polyethersulfone membrane filter (PES)*. For recovery of test spores, 47 mm diameter and 0.2 µm pore size. Any filtration apparatus may be used including filtration units (reusable or disposable). h. *Vials* with lids (plastic or comparable). Sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), approximately 20 mL capacity, for holding inoculated carriers to be exposed to the test chemical and for accommodating neutralizer. i. *Vortex mixer*. To vortex the fluid in the vials to ensure efficient recovery of the test organism. j. *Certified timer*. Readable in minutes and seconds. k. *Desiccator* (with gauge to measure vacuum) with fresh desiccant (e.g., $CaCO_3$). For drying the inoculum on the carriers. l. *Vacuum source*: In-house line or suitable vacuum pump (0.068 to 0.085 MPa) for drying carriers and for membrane filtration. m. *Microscope*. With 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option. To examine spores. n. *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. Use to ensure anaerobic environment. i. Alternatively, an activated anaerobic jar may be used according to manufacturer's instructions in place of the anaerobic chamber. o. *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 incubator at 36±1°C. p. *Digital Titrator kit*. To measure total chlorine and water hardness. Alternate titration methods may be used. q. *Laboratory film or sterile bags (18 by 30 cm or equivalent)*. To retain moisture in plates during prolonged incubation in an anaerobic chamber.
12. Procedure and Analysis	1. In brief, the method uses disks (1 cm in diameter) of brushed stainless steel to represent hard, non-porous surfaces. Each disk receives 10 µL of the final test suspension. The final test suspension is dried and exposed to 50 µL of the test chemical (treated carriers) or 50 µL of a control fluid (control carriers). The contact time is allowed to elapse and an appropriate neutralizer is added at the end of the contact time. The neutralized carriers

	are vortexed and the resulting suspension is serially diluted and filtered to determine the presence of spores. Based on mean $\log_{10}$ density values, the LR in the viability of the test organism on treated carriers is calculated in relation to the viability count on the control carriers. 2. With each efficacy test, three inoculated carriers are exposed to a treatment consisting of 50 µL of 1,500±150 ppm NaOCl. The mean LR (<3.0) in the viability of the spores on test system control carriers ensures the validity of the data.
12.1 Culture/ Inoculum Preparation	a. Prepare spores of *C. difficile* (ATCC 43598) according to MLB SOP MB-28 (based on ASTM E2839). i. Spores may be stored at -80±5°C for up to 90 days prior to use. ii. The mean LD of spores for control carriers is 6.0 to 7.0 spores/carrier, with each control carrier exhibiting a LD of 6.0 to 7.0.
12.2 Preparation and sterilization of carriers	a. Without magnification, visually check the brushed top surface of the carriers for abnormalities (e.g., rust, chipping, atypical brushed striations) and discard if observed; refer to Figures 1 and 2 for examples of typical acceptable and unacceptable carriers, respectively. b. Soak visually screened carriers in a suitable laboratory detergent solution free from any antimicrobial activity for 2-4 h to degrease and then rinse thoroughly in distilled or deionized water. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting. c. Using gloved hands or forceps, 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), ensure carriers were not damaged (scratched) during processing. d. Cover the Petri dish with its lid and sterilize by autoclaving for 45 min at 121°C on a gravity cycle. i. Alternative validated sterilization cycles may be used to sterilize carriers. ii. Place Petri dish with carriers in autoclave pouch for sterilization. e. Visually inspect carriers to ensure that they are dry following sterilization.

	f. After sterilization, aseptically transfer carriers with forceps to sterile plastic Petri dishes without filter paper for inoculation. g. Sterilized carriers may be stored and used for up to six months.[1]
12.3 Final Test Suspension Preparation	a. Defrost a cryovial of the qualified spore suspension at room temperature. Each cryovial is single use only. b. Vortex the thawed spore suspension for 45-60 s to resuspend the spores. c. Add the spore suspension to the three-part soil load. i. To obtain 500 µL of the final test suspension, vortex each component and combine the following (or appropriate ratio): 25 µL BSA stock, 35 µL yeast extract stock, 100 µL mucin stock, and 340 µL spore suspension.
12.4 Carrier Inoculation	a. Following the combination of the spore suspension and the soil load, vortex-mix the final test suspension for approximately 10 s; use within 30 min for carrier inoculation. b. For carrier inoculation: i. Withdraw 10 µL of the final test suspension with a calibrated positive-displacement pipette (with a 10 µL pipette tip) and deposit the final test suspension in the center of each carrier. ii. Inoculate a sufficient number of carriers for testing (for example, ten carriers exposed per test chemical/concentration/contact time combination, three exposed to the test system control, three control carriers, plus extras [for example, three extra carriers]). iii. Vortex-mix the final test suspension for approximately 5 s after inoculating every 5 carriers. iv. When inoculating, avoid contact of pipette tip with the carrier; do not spread the final test suspension with the pipette tip. v. The same pipette tip may be used to inoculate each batch of carriers. Discard any inoculated carrier where the final test suspension has run over the edge.

[1] Not in ASTM E3218-21.

	c. Dry the carriers inside a plastic Petri plate (up to 15 carriers/Petri plate) with the lid off in a biological safety cabinet (BSC) (up to 60 min or until the inoculum is visibly dry). i. After the inoculum has dried, place the Petri plate without the lid in a desiccator connected to a vacuum line. ii. Cover the desiccator and make sure that it is properly sealed. Continue drying the carriers (with the lid off the Petri plate) under vacuum maintained at 0.068 to 0.085 MPa for 120±5 min at room temperature. d. At the end of the drying period, turn off the vacuum, and cover the plate. Observe the dried inoculum on each carrier. Refer to Fig. 3 for an example of a typical dried carrier. i. Discard any carrier on which the inoculum has dried near the edge of the carrier or has run off of the surface. ii. Use inoculated carriers immediately or store the inoculated carriers in the desiccator without vacuum. Use dried carriers within 24 h of inoculation.
12.5 Preparation of test system control	a. Use sterile deionized water as the diluent to prepare 1,500±150 ppm solution of reagent grade sodium hypochlorite. b. Verify the concentration of the prepared NaOCl solution using an appropriate titration procedure (e.g., Hach digital titrator) prior to use and use the NaOCl solution within 3 h of preparation.
12.6 Prepare test chemical	a. When preparing the test chemical, ensure that the test chemical is adequately mixed. Use within 3 h of preparation or as specified in the manufacturer's instructions. b. Measuring error increases as delivery volume decreases. To minimize variability due to measuring error, a minimum of 1.0 mL or 1.0 g of concentrated test chemical should be used when preparing use-dilutions for testing. Use v/v dilutions for liquid test chemicals and w/v dilutions for solid test chemicals. Refer to MLB SOP MB-22 for additional information. c. Evaluate the test chemical at room temperature (22±2 °C). If necessary, place test chemical in water bath prior to use to equilibrate to the appropriate temperature for approximately 10 min. Record temperature.

12.7 Efficacy evaluation – treated carriers	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. In a timed fashion at pre-determined staggered intervals, deposit 50 µL of the test chemical (treatment) over the dried inoculum on the carriers, ensuring complete coverage of the inoculum. i. Use a new tip for each carrier; do not touch the pipette tip to the carrier surface. ii. During testing, do not process carriers where the test substance runs off of the carrier; replace with new carrier(s) and vial(s) if this occurs. iii. Do not cap the vials. c. Hold carriers at 22±2°C for specified contact time. d. Within ±3 s of the end of the contact period, add 10 mL of neutralizer at room temperature to each vial in the specified order according to the predetermined staggered intervals. e. Cap the vial and briefly vortex (2 to 3 s). The neutralized vial is the 10^0 dilution. f. Following the neutralization of the entire set of carriers, vortex each vial for 30±5 s at high speed to recover the inoculum; ensure that the carrier is vortexing along with the liquid in the vial. g. Visually examine each carrier (that is, look at the carrier through the bottom of the vial) and, in case of incomplete inoculum removal, perform further vortexing (for example, 30±5 s) to remove inoculum. Do not remove the carrier from the vial.
12.8 Dilution and Recovery	a. Vortex-mix the vial (10^0 dilution) for approximately 5 s and prepare serial ten-fold dilutions in PBS-T as necessary to achieve countable colonies in the target range of 20 to 200 CFU on the filters. Initiate dilutions within 30 min of neutralization. b. For treated carriers, filter the entire contents of the vial (10^0 dilution) through a 0.2 µm PES membrane filter; the entire contents of other dilutions may be filtered as necessary. Initiate filtration within 30 min of preparing the dilutions. c. Prior to filtration, pre-wet each membrane filter with approximately 10 mL PBS; apply vacuum to filter the contents. Leave the vacuum on for the duration of the filtration process.

i. To filter the contents of the vial, vortex-mix contents (5 to 10 s) and pour the contents into a filter unit.

ii. Rinse the vial with approximately 20 mL of PBS, vortex-mix for approximately 5 s and pour the entire contents of the vial into the same filter unit. Rinse the inside surface of each filter unit with an additional approximately 20 mL PBS.

iii. Gently decant the liquid from the vial into the filter unit, retaining the carrier in the vial.

iv. If a carrier falls onto the filter membrane, aseptically remove it using sterile forceps.

v. If the filter membrane is compromised (for example, punctured) by a fallen carrier, discard the filter membrane and repeat the test chemical exposure using an extra carrier.

d. To filter the entire contents of dilution tubes, vortex-mix the tube for approximately 5 s and pour into the filter.

i. Rinse each tube once with approximately 10 mL of PBS, vortex-mix for approximately 5 s, and pour the contents of the tube into the same filter unit.

ii. Rinse the inside surface of each filter unit with an additional approximately 20 mL PBS.

e. Aseptically remove the membrane filters in order (test chemical, test system control, controls) and place on the pre-reduced BHIY-HT.

i. Open each sealed package of BHIY-HT plates just prior to placement of the membrane filter.

ii. Avoid trapping any air bubbles between the membrane filter and the agar surface; use sterile forceps to reposition the filter if necessary.

f. At the end of the testing, filter approximately 20 mL of the PBS-T and approximately 20 mL of the PBS used in the test using two separate membrane filters to assess reagent sterility.

g. Place BHIY-HT plates with membrane filters under anaerobic conditions within 60 min of opening the package of plates.

i. Examine the plates immediately prior to use. Do not use plates showing signs of darkening, bubbles, or other color abnormalities.

	h. Incubate BHIY-HT plates with membrane filters under anaerobic conditions at 36±1°C for 120±4 h. i. If using an anaerobic chamber, bag or seal plates with laboratory film after approximately 24 h of incubation to minimize moisture loss. ii. Bagging or sealing plates with laboratory film immediately upon incubation inhibits growth of the organism due to the presence of oxygen absorbed into the agar plate during the placement of membrane filters. i. At the end of the incubation period, count the CFU on each filter. j. Ensure the sterility of the reagents (PBS-T and PBS). If sterility is not observed, repeat testing with fresh, sterile reagents. k. Observe the colony characteristics from at least one of the filters for purity and typical characteristics of the test microbe. i. On white filters, *C. difficile* colonies appear yellow-brown to tan. ii. On BHIY-HT after 48 to 120 h of incubation, *C. difficile* colonies appear circular with an entire edge, convex, smooth and gray. iii. Inspect growth from a typical colony under phase contrast microscopy. A colony may be comprised of both spores and vegetative cells: under phase contrast microscopy, spores appear bright and ovular while vegetative cells appear dark and rod-shaped.
12.9 Test system control	a. Use sterile deionized water as the diluent to prepare a 1500 ± 150 ppm solution of reagent grade sodium hypochlorite. b. Verify the concentration of the prepared NaOCl solution using an appropriate titration procedure prior to use and use the NaOCl solution within 3 h of preparation. c. On each test day, expose each of the three carriers to 50 µL of the test system control (1500±150 ppm NaOCl) and evaluate as described in 12.7 through 12.8 using a 3 min ± 3 s contact time. d. Neutralize test system control carriers with 10 mL PBS-T with 0.1 % (w/v) sodium thiosulfate. e. The test system control must exhibit a mean LR <3.0 spores/carrier for a valid test.

	f. For test system control carriers, prepare serial dilutions out to 10^{-5} and filter the entire contents of the 10^{-3}, 10^{-4}, and 10^{-5} dilution tubes.
12.10 Control carrier counts	a. On each test day, expose each of the three control carriers to 50 µL of PBS-T and evaluate the carriers as described in 12.7 through 12.8. i. Expose control carriers to PBS-T for the same contact time used for the treated carriers. ii. Neutralize control carriers with the same neutralizer used for the treated carriers. b. For control carriers, prepare serial ten-fold dilutions out to 10^{-5} and filter the entire contents of the 10^{-4} and 10^{-5} dilution tubes.
12.11 Results	a. For all carriers (test chemical treatment, test system control, and control carrier counts), count the appropriate number of CFU (for example, up to 200 CFU for filters). Use CFU to calculate log reduction. Log reduction is used to determine test chemical effectiveness.
13. Data Analysis/ Calculations	1. Use values with at least three significant figures when performing calculations. Report log reduction values with at least two significant figures. 2. The $\log_{10}$ density (LD) for each treated, test system control, and control carrier is calculated as follows: $$Log_{10}\left\{\left[\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(C_i \times D_i)}\right] \times V\right\}$$ where: Y = CFU per filter, C = volume filtered, V = total volume of neutralizer, $D = 10^{-k}$, k = dilution, n = number of dilutions, and i = lower limit of summation (the fewest number of dilutions). 3. Calculate the mean LD for three test system control carriers and three control carriers as follows: Mean LD = [Log_{10}(carrier 1) + Log_{10}(carrier 2) + Log_{10}(carrier 3)]/3 4. Calculate the mean LD for each set of ten treated carriers as follows: Mean LD = [Log_{10}(carrier 1) + Log_{10}(carrier 2) + Log_{10}(carrier 3) + Log_{10}(carrier 4) + Log_{10}(carrier 5) + Log_{10}(carrier 6) + Log_{10}(carrier 7) + Log_{10}(carrier 8) + Log_{10}(carrier 9) + Log_{10}(carrier 10)]/10

	a. For the purpose of calculation, if no organism is recovered from a test carrier, the log density for that carrier is 0 provided that the entire contents of the 10^0 dilution was filtered. 5. Calculate the $\log_{10}$ reduction (LR) for the test system control: LR= MeanLD(Control Carriers) –MeanLD(Test System Control Carriers) 6. Calculate the LR for each test chemical as follows: LR= MeanLD(Control Carriers) –MeanLD(Treated Carriers) a. If no organism is recovered from each of the ten test carriers, the log reduction is greater than or equal to the mean control carrier log density.
14. Forms and Data Sheets	1. Attachment 1: Carrier Specifications 2. Attachment 2: Neutralizer Verification Test 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record Form — MB-03_F1.docx QM for *C.d.*: Test Information Sheet — MB-31-07_F1.docx QM for *C.d.*: Time Recording, Dilution and Filtration Sheet — MB-31-07_F2.docx QM for *C.d.*: Results Sheet — MB-31-07_F3.docx QM for *C.d.*: Test Microbe Confirmation Sheet — MB-31-07_F4.docx QM for *C.d.*: Processing Sheet — MB-31-07_F5.docx QM for *C.d.* Neutralization Verification Test: Test Information Sheet — MB-31-07_F6.docx QM for *C.d.* Neutralization Verification Test: Test Suspension Preparation Sheet — MB-31-07_F7.docx QM for *C.d.* Neutralization Verification Test: Processing Sheet — MB-31-07_F8.docx QM for *C.d.* Neutralization Verification Test: Time Recording and Results Sheet — MB-31-07_F9.docx
15. References	1. ASTM E3218-21, Standard Test Method for Quantitative Method for Testing Antimicrobial Agents against Spores of *C. difficile* on Hard, Nonporous Surfaces. ASTM International, West Conshohocken, PA, 2021.

Attachment 1

Carrier Specifications

(AISI Type 304 Stainless Steel Carriers)

General Description: 1 cm non-magnetic disc made of AISI Type 304 Stainless Steel (SS) with 150 grit unidirectional brushed finish on one side.

Material: AISI Type 304 Austensic stainless steel consisting of 18% to 20% Chromium, 8% to 10.5% Nickel, and a maximum of 0.8% Carbon.

- European Specification X5CrNi18-10 Number 1.4301
- Japanese Specification: JIS 4303 SUS 304

Carrier Dimensions:

- Diameter: 1cm (±0.5 mm)
- Stainless Steel Sheet Thickness: 22 gauge; carrier manufacturer will provide thickness of the original stainless steel sheet (in mm).
- Flatness: Carrier height not to exceed 110% of the thickness of the uncut sheet of stainless steel from which the carriers are manufactured.

Finish: A ground unidirectional finish obtained with 150 grit abrasive (AISI) on the top side of the stainless steel sheet.

Burr Removal: Remove burrs from the edges of the discs on the bottom side of the carrier using a manual process.

Passivation: Parts are passivated by the carrier manufacturer according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus-based degreaser by soaking in the degrease solution for 1 hour
- Rinse with de-ionized water
- Passivate by soaking carriers:
    - 7% citric acid solution
    - 20-30 min at 35±5°C.
- Rinse with de-ionized water
- Air dry

Examples of Physically Screened Carriers[2]

Fig. 1: Examples of typical acceptable carriers.

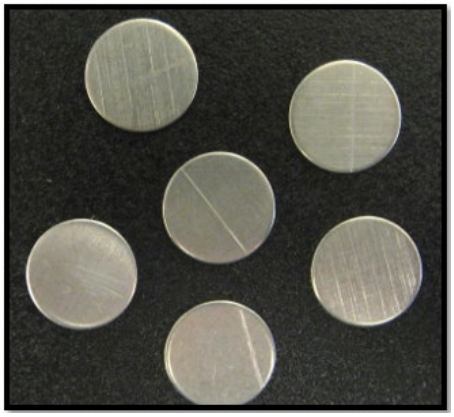

Fig. 2: Examples of typical unacceptable carriers.

A B

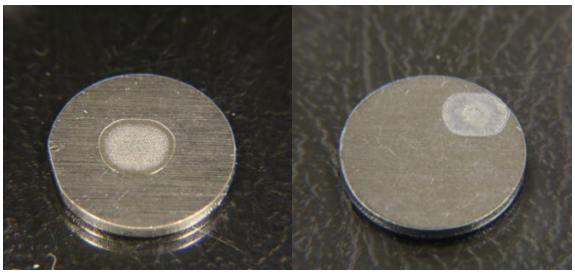

Fig. 3: Example of an acceptable (A) and unacceptable inoculated carrier (B).

[2] Carriers are screened without magnification.

Attachment 2

Neutralization Verification Test

1. Prepare *Spore Suspension A* (without soil load)
   a. Defrost a cryovial of *C. difficile* stored at -80±5°C. Vortex the thawed spore suspension for 45-60 s.
   b. Dilute the spore suspension with PBS-T to achieve an average challenge of 20-200 CFU per 10 µL (e.g., serially dilute spores through 10^{-5}).
   c. Use *Spore Suspension A* within 30 min of preparation.
2. Prepare *Final Spore Suspension B* (with soil load).
   a. Prepare the soil load: vortex each component for 10 s and combine 25 µL BSA, 35 µL yeast extract, 100 µL of mucin and 340 µL of *Spore Suspension A* from dilutions 10^{-4} and 10^{-5}. Vortex-mix for 10-15 s.
   b. Note: Use two separate serial dilutions of *Spore Suspension A* (10^{-4} and 10^{-5}) to prepare two different concentrations of *Final Spore Suspension B* to ensure there is at least one dilution with an average challenge of 20-200 CFU.
3. Treatments
   a. *Neutralizer Effectiveness*: Add 50 µL of the test chemical to each of three reaction vessels. At timed intervals, add 10 mL of neutralizer to each tube and briefly swirl (by hand). After 10 s, add 10 µL of *Final Spore Suspension B* to each tube and briefly vortex (5 s). Proceed with section 4.
   b. *Neutralizer Toxicity Control*. Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add 10 µL of *Final Test Suspension B* to each tube and briefly vortex (5 s). Proceed with section 4.
   c. *Titer Control*. Add 10 mL PBS-T to each of three reaction vessels. At timed intervals, add 10 µL of *Final Spore Suspension B* to each tube and briefly vortex (5 s). Proceed with section 4.
4. Processing and Recovery
   a. Hold the mixture for 10±1 min at room temperature (22±2°C). Conduct steps (e.g., addition of organism, neutralizer) at timed intervals (e.g., 30 s) to ensure consistent time of contact.
   b. At the conclusion of the holding period, vortex-mix each tube for 5-10 s and pass the entire contents of each mixture through a separate, pre-wetted 0.2 µm PES membrane filter.

c. Wash each tube with approximately 10 mL PBS and vortex-mix for 5-10 s; filter the wash through the same filter membrane. Finish the filtration process by rinsing the inside of the funnel unit with about 20 mL of PBS, filtering the rinsing liquid through the same filter membrane. Initiate filtration as soon as possible (e.g., within 30 min).

d. Aseptically remove the membrane with sterile forceps and place it carefully over the surface of the recovery medium (BHIY-HT). Avoid trapping air bubbles between the filter and the agar surface. Incubate the plates anaerobically for 120±4 hours at 36±1°C. If using an anaerobic chamber, bag or seal plates with laboratory film after approximately 24 h of incubation to minimize moisture loss.

5. Acceptance Criteria

a. The number of CFU in the *Titer Control* should be in the range of 20-200 CFU/filter.

b. Calculate the mean CFU per filter for each treatment. For calculations described in section 5c and 5d below, use a dilution for which the titer control resulted in 20-200 CFU/filter.

c. The recovered number of CFU in the *Neutralizer Effectiveness* treatment is at least 50% of the *Titer Control*; this verifies effective neutralization. A count lower than 50% indicates that the neutralizer is not adequate to inactivate the active ingredient (test chemical). Counts higher than the *Titer Control* are also valid.

d. The recovered number of CFU in the *Neutralizer Toxicity Control* is at least 50% of the *Titer Control*. A count of lower than 50% indicates that the neutralizer is harmful to the test organism. Counts higher than the *Titer Control* are also valid.