US Environmental Protection Agency
Office of Pesticide Programs

EPA MLB SOP-MB-31: Procedure for the OECD Quantitative Method for Testing Antimicrobial Products against Spores of *Clostridium difficile* (ATCC 43598) on Inanimate, Hard, Non-porous Surfaces

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OECD Quantitative Method for Testing Antimicrobial Products against Spores of *Clostridium difficile* (ATCC 43598) on Inanimate, Hard, Non-porous Surfaces.

I. Overview

A. This document describes a quantitative method intended for evaluating the sporicidal efficacy of liquid disinfectants against spores of *Clostridium difficile* (ATCC 43598) on inanimate, hard, non-porous surfaces.

A spore suspension should be developed and qualified according to 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 before an efficacy evaluation can be performed using this method.

Verify the neutralizer specified for the product in advance of product testing. See Attachment 2.

B. This method is based on an Organisation for Economic Co-operation and Development (OECD) Guidance Document, dated June 21, 2013 (see reference A); the protocol incorporates methodologies specific to testing spores of *C. difficile*.

C. Log_{10} Reduction (LR) of viable spores of *C. difficile* is the quantitative measure of efficacy for liquid disinfectants.

D. In brief, the method uses disks (1 cm in diameter) of brushed stainless steel to represent a hard, non-porous surface. Each disk receives 10 µL of spore suspension. The inoculum is dried and exposed to 50 µL of the test substance; control carriers receive an equivalent volume of a control fluid. 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 viable spores. Based on mean log 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.

E. When a product is evaluated for efficacy testing against *C. difficile* using the OECD method, a solution of 1,500±150 ppm sodium hypochlorite (NaOCl) is included as a test system control.

II. Data Requirements

A. For an acceptable test, the log density (LD) for each control carrier should be 10^6 - 10^7 viable spores/carrier. The 1,500 ppm NaOCl test system control should exhibit an LR of <3 in viable spores using a contact period of 3 min±3 s.

B. Retesting guidance.

Outcome Scenario	Passed/Failed[1] Test	Lot Retest Permitted?
Control carrier counts above acceptable range	Failed Test	Yes
Control carrier counts above acceptable range	Passed Test	Not needed
Control carrier counts below acceptable range	Failed Test	No

Control carrier counts below acceptable range	Passed Test	Yes

[1]Failed tests are defined as tests that do not meet the *C. difficile* performance standard for the efficacy claim. The performance standard is outlined in the most current *C. difficile* guidance document.

III. Special Apparatus and Materials

A. Test microbe. *C. difficile* (ATCC 43598); spores prepared according to EPA MLB SOP MB-28: Production and Storage of Spores of *Clostridium difficile* for Use in the Efficacy Evaluation of Antimicrobial Agents.

B. Recovery medium

1. *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. *Neutralizer.* The neutralizer for the product is specified by the manufacturer.

6. *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.

7. *Test substance.* Antimicrobial test solution.

8. *Test substance diluent.* The test substance diluent as specified by the manufacturer.

9. *Laboratory Grade Sodium Hypochlorite with total chlorine ≥4%.* To prepare 1,500±150 ppm total chlorine for test system control.

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

11. *Tween-80* (polysorbate 80). To make PBS-T.

12. *Liquinox (1% solution) or equivalent.* To clean carriers.

D. Apparatus

1. *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). Disks (1 cm in diameter) made from 0.8 mm thick sheets of brushed stainless steel (AISI #304) (Pegen Industries, part #304-107) may be used instead of 430 stainless steel carriers for product performance testing of oxidative chemistries such as peracetic acid and peroxides.

   i. 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 specifications.

   ii. Use 430 stainless steel carriers for the control carriers and the 1,500 ppm NaOCl test system control.

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

3. *Calibrated micropipettes* (e.g., 200 µL) with 10-100 or 20-200 µL tips. For deposition of test substance on carrier.

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

5. *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.

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

7. *Magnet.* To hold magnetic carriers in place in the vial while the liquid is being dispensed into membrane.

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

9. *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.

10. *Vortex mixer*. To vortex the eluate and rinsing fluid in the carrier vials to ensure efficient recovery of the test organism(s).

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

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

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

14. *Microscope*. With 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option. To examine spores.

15. *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.

16. *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.

17. *HACH's Digital Titrator kit*. To measure total chlorine and water hardness.

IV. Procedure and Analysis

A. For each product test, evaluate 10 inoculated carriers with the product, 3 inoculated carriers with 1,500±150 ppm NaOCl (test system control), and 3 inoculated control carriers with PBS-T. For conducting the carrier-based NaOCl testing to qualify spore suspension, refer to section IV.G-H of the spore production method, SOP MB-28.

B. Preparation and sterilization of carriers

1. Without magnification, visually check the brushed top surface of the carriers (with the rounded edge) 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.

2. Soak visually screened carriers in a suitable detergent solution (e.g., Liquinox) free from any antimicrobial activity for 2-4 h to degrease and then rinse thoroughly in distilled water. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.

3. 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 its lid and sterilize by autoclaving for 45 min at 121°C on a gravity cycle. After sterilization, aseptically transfer carriers to sterile plastic Petri dishes without filter paper for inoculation.

4. Sterilized carriers may be stored and used for up to six months.

C. Preparation of final spore suspension (with soil load)

1. Defrost a cryovial (at -80±5°C) of the qualified spore suspension at room temperature. Each cryovial is single use only.

2. Vortex the thawed spore suspension for 45-60 s to resuspend spores.

3. Add OECD three-part soil load to spore suspension. To obtain 500 µL of the final test suspension with soil load, vortex each component and combine the following (or appropriate ratio):

   i. 25 µL BSA stock

   ii. 35 µL yeast extract stock

   iii. 100 µL mucin stock

   iv. 340 µL spore suspension

4. Following the addition of the soil load, vortex mix for approximately 10 s and use within 30 min for carrier inoculation.

D. Inoculation and drying of carriers

1. 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. Inoculate a minimum of eighteen carriers (use ten carriers for product, three for 1,500 ppm NaOCl test system control, three for control carriers, and two extras). Vortex-mix the final spore suspension for approximately 5 s after inoculating every 5 carriers.

2. Avoid contact with carrier and do not spread the spore suspension with the pipette tip. The same pipette tip may be used to inoculate each batch of carriers. Discard any inoculated carrier where the final spore suspension has run over the edge.

3. Dry the carriers inside a plastic Petri plate (up to 15 carriers/Petri plate with the lid off) in the biological safety cabinet (BSC) for 30-50 min or until the inoculum has dried. After the inoculum has dried, place the Petri plate in a desiccator connected to a vacuum line. Remove the Petri plate lid. Cover the desiccator and make sure that it is properly sealed. Continue drying the carrier under vacuum for 120±5 min at room temperature inside the BSC.

4. 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. Use inoculated carriers immediately or store the inoculated carriers in the desiccator without vacuum. Use dried carriers within 24 h of inoculation.

E. Preparation of test control system

1. Using sterile deionized water as the diluent, prepare a 1,500±150 ppm solution of NaOCl using laboratory grade sodium hypochlorite.

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

F. Disinfectant sample preparation

1. Use the test substance within three hours of preparation unless test parameters specify otherwise. Record the time of test substance preparation. For dilutable concentrates, use the diluent that is consistent with that used for the sterilant, sporicide, or hospital disinfectant claim.

G. Exposure of the dried inoculum to the test substance, 1,500 ppm NaOCl (test system control), or PBS-T (control counts)

1. 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.

2. Use a certified timer to ensure that each carrier receives the required exposure time.

3. In a timed fashion, deposit 50 µL of the test substance (carriers 1 to 10), and 1,500 ppm NaOCl (carriers 11 to 13), equilibrated to 22±2°C, over the dried inoculum on 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.

4. Hold carriers at 22±2°C for specified contact time for the product and 3 min±3 s for test system control.

5. Treat control carriers last – each control carrier receives 50 µL PBS-T, equilibrated to 22±2°C, instead of the test substance. Hold the control carriers at 22±2°C for the same contact period as the product.

H. Neutralization of test substance and elution of test organism

1. Use the same neutralizer for the control carriers and the treated carriers. The neutralizer for the 1,500 ppm NaOCl is PBS-T with 0.1% (w/v) sodium thiosulfate.

2. 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, including controls,

according to the predetermined schedule. Cap the vial and briefly vortex (2-3 s). The neutralized vial is the 10^0 dilution.

3. 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. Visually examine each carrier and, in case of incomplete elution, perform further vortexing (30±5 s) to remove inoculum. Do not remove the carrier from the vial.

I. Dilution and recovery

1. Initiate dilutions within 30 min at room temperature after neutralization. Initiate filtration within 30 min of preparing the dilutions. Do not direct plate.

2. Process the product carriers first. Prior to filtering the contents of tubes and vials, pre-wet each membrane filter with approximately 10 mL of sterile PBS.

3. For product carriers, vortex-mix the vial (10^0) for approximately 5 s and remove 1 mL to prepare serial dilutions in 9 mL PBS-T out to a minimum of the 10^{-1} dilution. Filter the contents of the 10^0 and 10^{-1} dilutions. For the 1,500 ppm NaOCl test system control, prepare serial dilutions out to 10^{-5} and filter the contents of the 10^{-3}, 10^{-4}, and 10^{-5} dilution tubes.

4. For filtration of contents in dilution tubes, vortex-mix for approximately 5 s and pour into filter. Rinse each tube once with ~10 mL of PBS, vortex-mix for approximately 5 s, 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.

5. For filtering the vial, vortex-mix contents (5-10 s) 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, vortex-mix for approximately 5 s, and while keeping the magnet in place, pour the wash liquid into the same filter unit. 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 ~20 mL PBS.

6. 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. Filter the contents of the 10^{-4} and 10^{-5} dilution tubes.

7. Aseptically remove the membrane filter (product, 1,500 ppm NaOCl, followed by the controls) and place on the pre-reduced BHIY-HT. Open each sealed package of BHIY-HT plates just prior to placement of the membrane filter. Avoid trapping any air bubbles between the membrane filter and the agar surface.

8. At the end of the testing, filter approximately 20 mL of the PBS-T and 20 mL of the PBS used in the test using two separate membrane filters.

9. Place BHIY-HT plates with membrane filters under anaerobic conditions within 60 min of opening the package of plates. Incubate BHIY-HT plates with membrane filters under anaerobic conditions at 36±1°C for 120±4 h. If using an anaerobic chamber, bag or seal plates with Parafilm after approximately 24 h of incubation to minimize moisture loss.

J. Results

1. At the end of the incubation period, remove plates from the anaerobic chamber or jars and count the colonies on each of the filters. Record as Colony Forming Units (CFUs) per filter.

2. Record colony counts in excess of 200 CFU per filter as Too Numerous to Count (TNTC). If no colonies are present, record as zero.

3. Ensure the sterility of the reagents (e.g., PBS-T and PBS). If sterility is not observed, invalidate the test, and repeat testing with fresh sterile reagents.

4. Observe the cultural characteristics of CFUs from one of the filters for purity and typical characteristics of the test microbe (see Table 1). Inspect growth from a typical CFU under phase contrast microscopy. If necessary, conduct additional biochemical and antigenic analyses or other comparable confirmatory procedures (e.g., Vitek) for verification of the test organism. Record results.

5. For the 1,500 ppm NaOCl test system control, exhibit a LR of <3 in viable spores using a contact period of 3 min±3 s.

6. 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.

*At 48-120 h

V. Data Analysis and Calculations

A. Colony counts (CFU) at each dilution are used to calculate the log reduction in viable spores.

B. Use values with at least two significant figures when performing calculations (e.g., log density, mean log density). Report the final mean log reduction value with one significant figure (e.g., round up to the nearest tenth).

C. To calculate the CFU/treated carrier use the following equation:

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

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 of medium

originally in the vial with the carrier (10 mL). Account for the volume filtered in calculations.

1. 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.

D. Calculate the log density (LD) of each carrier by taking the log of the CFU/carrier.

E. Calculate the mean log density across treated carriers with product.

F. Calculate the mean log density across control carriers.

G. Calculate the mean log reduction (LR) for treated carriers:

LR= mean log density for control – mean log density for treated

H. For a set of 10 treated carriers: when the 10^0 dilution (the contents of the vial with the carrier) is filtered 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.

I. Calculate the mean LR for the carriers treated with 1,500 ppm NaOCl test system control:

LR= mean log density for control – mean log density for 1,500 ppm NaOCl.

VI. Attachment

A. Attachment 1: Carrier Specifications

B. Attachment 2: Neutralization Verification Test

C. Attachment 3: Gravimetric and Physical Wetness Determination for Towelettes.

VII. Reference

A. OECD Guidance Document dated June 21, 2013. Quantitative Method for Evaluating Bactericidal Activity of Microbicides used on Hard Non- Porous Surfaces.

Attachment 1

Carrier Specifications

AISI Type 430 Stainless Steel Carriers

General Description: 1 cm magnetic disc made of AISI Type 430 Stainless Steel (SS) with No.4 finish on one side and rounded edges on top side.

Material: AISI Type 430 Ferretic stainless steel consisting of 16% to 18% Chromium, a maximum of 0.5% Nickel and a maximum of 0.12% Carbon.

- European Specification X6Cr17 Number 1.4016
- Japanese Specification: JIS G4305; EN10088-2

Dimensions:

- Diameter: 1cm (0.39”) in diameter
- Thickness: 0.8 mm (22 gauge / 0.031”)
- Flatness: some concavity desired at edges

Finish

No. 4 Finish is produced with short, parallel polishing lines. The final finish can be anywhere between 120 and 320 grit.

Tumbling

To remove burrs from the edges of the discs they are tumble deburred in a vibratory tumbler using ceramic median and cleanser. Tumbling time is dependent on the extent to which burring occurs.

Passivation

Parts are passivated according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus based degreaser
- Rinse with tap water
- Passivate
    - 7% Citric Acid Solution
    - Minimum of 20 min at 20-50°C.
- Rinse with de-ionized water
- Air dry

AISI Type 304 Stainless Steel Carriers

General Description: 1 cm non-magnetic disc made of AISI Type 304 Stainless Steel (SS) with No.4 finish on one side and rounded edges on top 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

Dimensions:

- Diameter: 1cm (0.39”) in diameter
- Thickness: 0.8 mm (22 gauge / 0.031”)
- Flatness: some concavity desired at edges

Finish

No. 4 Finish is produced with short, parallel polishing lines. The final finish can be anywhere between 120 and 320 grit.

Tumbling

To remove burrs from the edges of the discs they are tumble deburred in a vibratory tumbler using ceramic median and cleanser. Tumbling time is dependent on the extent to which burring occurs.

Passivation

Parts are passivated according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus based degreaser
- Rinse with tap water
- Passivate
    - 7% Citric Acid Solution
    - Minimum of 20 min at 20-50°C.
- Rinse with de-ionized water
- Air dry

Examples of Physically Screened Carriers[1]

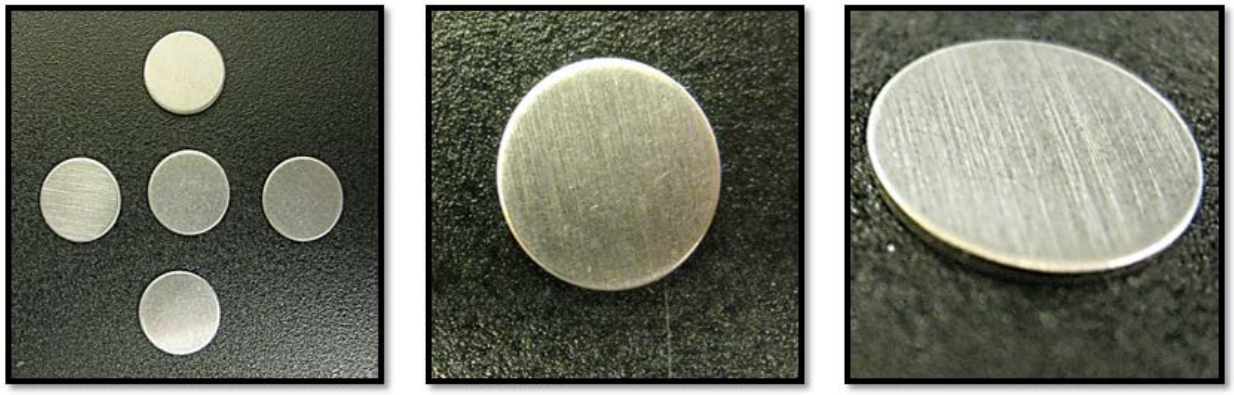

Fig. 1: Examples of typical acceptable 430 SS carriers.

[1]The same acceptance criteria used to screen the 430 SS carriers are used to screen the 304 SS carriers. Carriers are screened without magnification.

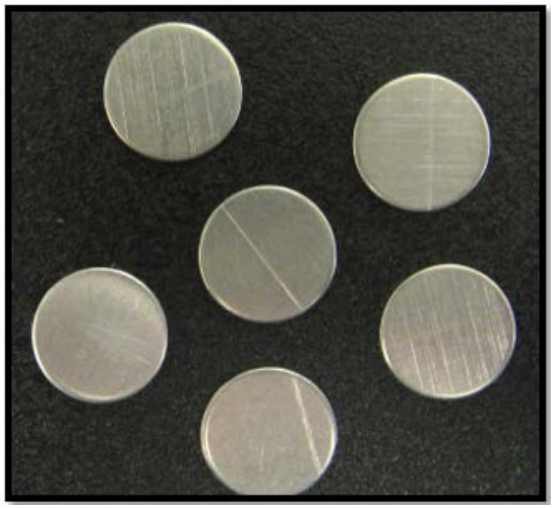

Fig. 2: Examples of typical unacceptable 430 SS carriers.

Attachment 2

Neutralization Verification Test

A. Preparation. Confirm the effectiveness of the neutralizer concurrently or prior to product testing.

1. Prepare spore suspension A (without soil load)

   i. Defrost a cryovial of *C. difficile* spores stored at -80°C. Vortex the thawed spore suspension for 45-60 s.

   ii. 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}).

   iii. Use spore suspension A within 4 hours of preparation.

2. Prepare final spore suspension B (with soil load).

   i. Prepare the soil load: vortex each component for 10 s and combine 12.5 µL BSA, 17.5 µL yeast extract, 50 µL of mucin and 170 µL of spore suspension A from dilutions10^{-4} and10^{-5}. Vortex-mix for 10-15 s.

   Note: Use two separate serial dilutions of spore suspension A (10^{-4} and10^{-5}) to prepare two different concentrations of final spore suspension B to ensure at least there is one dilution with an average challenge of 20-200 CFU.

B. Treatments

1. Treatment 1. *Neutralizer Effectiveness*: Add 50 µL of the test substance to each of three 15 mL test tubes. 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 processing and recovery.

2. Treatment 2: *Neutralizer Toxicity Control*. Add 10 mL neutralizer to each of three 15 mL test tubes. At timed intervals, add 10 µL of *Final Test Suspension B* to each tube and briefly vortex (5 s). Proceed with processing and recovery.

3. Treatment 3: *Titer Control*. Add 10 mL PBS-T to each of three 15 mL test tubes. At timed intervals, add 10 µL of final spore suspension B to each tube and briefly vortex for 5 s. Proceed with processing and recovery.

C. Processing and Recovery

1. 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.

2. At the conclusion of the holding period, vortex mix each tube for 5-10 s and pass each mixture through a separate, pre-wetted 0.2 µm PES membrane filter.

3. 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, filter the rinsing liquid through the same filter membrane. Initiate filtration as soon as possible (e.g., within 50 min).

4. Remove the membrane aseptically 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 Parafilm after approximately 24 h of incubation to minimize moisture loss.

D. Acceptance Criteria

1. For the assay to be considered valid, ensure that the recovered number of CFU in the Titer Control using Final Spore Suspension B yields 20-200 CFU per tube.

2. For determining and verifying the effectiveness of the neutralizer, ensure that:

   i. The recovered number of CFU in the Neutralizer Toxicity Control is at least 50% of the Titer Control. A count lower than 50% indicates that the neutralizer is harmful to the test organism. Note: counts higher than the Titer Control (e.g., 150% of the Titer Control) are also deemed valid.

   ii. The recovered number of CFU in the Neutralizer Effectiveness treatment is at least 50% of the Titer Control; this verifies effective neutralization. Note: counts higher than the Titer Control (e.g., 150% of the Titer Control) are also deemed valid.

Attachment 3

Gravimetric and Physical Wetness Determination

- For each batch, use three 150×20 mm glass Petri plates to represent the surface to be treated.
- Pre-clean each carrier surface with 70% ethanol, rinse in deionized water and air dry. Record the weight (weight #1: dry and untreated).
- Distribute the liquid in the canister or package, remove and discard the first 3-5 towelettes.
- For each carrier, remove one towelette from the container, unfold, and wipe the carrier surface in a circular fashion without lifting the towelette – treat up to the edge of the carrier.
- Re-weigh, record the results (weight #2: wet and treated).
- Allow carriers to sit horizontally for the contact time in an environmental chamber set at 35±5% relative humidity and 20-25°C.
- Record the final weight (weight #3: post contact time).
- Document the residual wetness by photograph or video.
- Use a single dry sheet of Kim Wipe (e.g., 11×21 cm) in the visualization of wetness, record observations.
- The data to show the presence of free-liquid on the treated surface by weight and physical observations (presence of wetness).