MICROBIOLOGICAL METHODS

Modification to the AOAC Sporicidal Activity of Disinfectants Test (Method 966.04): Collaborative Study

STEPHEN F. TOMASINO
U.S. Environmental Protection Agency, Office of Pesticide Programs, Microbiology Laboratory, Environmental Science Center, Ft. Meade, MD 20755-5350
MARTIN A. HAMILTON
Montana State University, Center for Biofilm Engineering, Bozeman, MT 59717-3980

Collaborators: M. Buen; H. Chan Myers; A. Garza; E. Gonzales; K. Kallander; A. Rodriguez; P. Stahnke; T. To

In an effort to improve AOAC Method 966.04, the Sporicidal Activity of Disinfectants Test, selected modifications to the procedure were evaluated in a collaborative study. Method 966.04 is used to generate efficacy data to support the product registration of sporicides and sterilants. The method is a carrier-based test that provides a qualitative measure of product efficacy against spores of *Bacillus subtilis* and *Clostridium sporogenes*. The use of garden soil extract and the lack of standard procedures for the enumeration of spores and neutralization of the test chemicals have been considered problematic for many years. The proposed modifications were limited to the *B. subtilis* and hard surface carrier (porcelain penicylinder) components of the method. The study included the evaluation of a replacement for soil extract nutrient broth and an establishment of a minimum spore titer per carrier, both considered crucial for the improvement and utilization of the method. Additionally, an alternative hard surface material and a neutralization confirmation procedure were evaluated. To determine the equivalence of the proposed alternatives to the standard method, 3 medium/carrier combinations, (*1*) soil extract nutrient broth/porcelain carrier (current method), (*2*) nutrient agar amended with 5 μg/mL manganese sulfate/porcelain carrier, and (*3*) nutrient agar amended with 5 μg/mL manganese sulfate/stainless steel carrier were analyzed for carrier counts, HCl resistance, efficacy, quantitative efficacy, and spore wash-off. The test chemicals used in the study represent 3 chemical classes and are commercially available antimicrobial liquid products: sodium hypochlorite (bleach), glutaraldehyde, and a combination of peracetic acid and hydrogen peroxide. Four laboratories participated in the study. The results of the spore titer per carrier, HCl resistance, efficacy, and wash-off studies demonstrate that amended nutrient agar in conjunction with the porcelain is comparable to the current method, soil extract nutrient broth/porcelain. The nutrient agar method is simple, inexpensive, reproducible, and provides an ample supply of high quality spores. Due to the current use of porcelain carriers for testing *C. sporogenes*, it is advisable to retain the use of porcelain carriers until stainless steel can be evaluated as a replacement carrier material for *Clostridium*. The evaluation of stainless steel for *Clostridium* has been initiated by the Study Director. Study Director recommendations for First Action revisions are provided in a modified method.

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Corresponding author's e-mail: tomasino.stephen@epa.gov

The U.S. Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) has responsibility for regulating antimicrobial products, including sporicides and sterilants, which are used to control pathogenic bacteria, viruses, and other microorganisms on inanimate surfaces. The U.S. Food and Drug Administration (FDA) Center for Devices and Radiological Health regulates antimicrobial chemicals (e.g., sterilants) used to process critical and semicritical medical devices. For the purpose of this paper, the terms sporicide and sterilant are considered as synonymous. In response to the intentional release of *Bacillus anthracis* spores in 2001 and the associated need for verifying the performance of sporicidal chemicals for building decontamination, OPP initiated a research program to evaluate and improve efficacy test methods for sporicides. One component of this effort involves the assessment of the AOAC Sporicidal Activity of Disinfectants, AOAC® *Official Method*℠ **966.04** (1), the method currently accepted by EPA and FDA for generating efficacy data to support the registration of sporicides (sterilants). AOAC Method **966.04**

is a carrier-based test that provides a qualitative measure of product efficacy against spores of *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 3584). For regulatory purposes, 60 carriers representing each of 2 types of surfaces (hard surface—porcelain penicylinders; porous surface—silk suture loops) are required to be tested against spores of both organisms on 3 product samples representing 3 different batches. Killing on all of the 720 carriers is an EPA regulatory requirement for sporicidal products.

The quality of the efficacy data generated with Method **966.04** has been considered problematic for many years. Potential sources of variability have been previously published, and several authors have proposed corrective solutions, including designing quantitative approaches to efficacy testing (2–6). The most significant concerns are associated with the qualitative nature of the method, the use of raw garden soil extract as a source of minerals for spore production, the carrier materials (unglazed porcelain and silk suture loops), the lack of a standardized procedure for enumeration of spores, spore wash-off, and the long incubation time (21 days). Recently, a modification to use polyester (Dacron®) loops instead of silk suture loops for testing peracetic acid-based sterilants was approved by the AOAC Methods Committee on Microbiology and Extraneous Materials—a First Action modification (7).

In this paper, we report on the conduct of a collaborative study to modify the method, present results, and provide recommendations. In addition, the modified Method **966.04**, including numerous editorial revisions, is presented. The order of discussion is based on the order of studies provided in the collaborative study protocol. Test methodology for each study component is largely reproduced from the actual collaborative study protocol with only minor changes in content and format.

Proposed Modifications—An Overview

Replacement for soil extract nutrient broth (SENB), a procedure modification.—In the current method, SENB is used for the cultivation (i.e., spore production) of *B. subtilis*; the procedure uses a nonstandardized extract of raw garden soil, is very complicated to prepare, and results in a highly variable preparation of spores (i.e., spore titer and quality). The use of a synthetic, standardized sporulation medium is highly recommended. Nutrient agar amended with 5 μg/mL manganese sulfate was evaluated as a replacement.

Alternative hard surface material (a procedure modification).—In Method **966.04**, unglazed porcelain (penicylinders) is used to represent a hard surface. Porcelain penicylinders are somewhat porous, easily damaged, and may become variable upon reuse. Stainless steel (penicylinders) was proposed as an alternate hard surface material. Stainless steel carriers, unlike porcelain, are easily cleaned and highly reusable, making them a viable option for replacement. In addition, the vendor who supplies the porcelain carriers, CeramTec (Laurens, SC), requires a 5000 piece minimum order. Fisher Scientific (Pittsburgh, PA), a vendor for stainless steel carriers, has a minimum order of 12 pieces. With stainless steel, the basic concept and carrier dimensions are retained, and the same carrier is used in the AOAC use-dilution methods (Methods **955.14**, **955.15**, and **964.02**).

Spore enumeration procedure and a target spore titer/carrier (a new procedure and standard).—Method **966.04** does not provide a procedure for measuring the number of spores per carrier (carrier counts). A carrier count procedure was provided and used to generate data to support the establishment of target carrier counts—a minimum of 1.0×10^5 ($\log_{10}$ density = 5.0) and a maximum of approximately 1.0×10^6 spores/carrier.

Neutralization confirmation (a new procedure).—Method **966.04** does not provide a procedure to confirm neutralizer effectiveness. The neutralization of active ingredients is one of the most important steps in efficacy testing of antimicrobial products. The selected neutralizer is used to stop the activity of the antimicrobial agent, a process essential to measuring effectiveness at a desired contact time, and allows for the recovery of viable inoculum. The neutralizer itself or in combination with the recovery medium must not exhibit bacteriostatic activity against the test microbe. A neutralization confirmation procedure that simulates Method **966.04** was proposed and conducted by 2 laboratories in this study.

Original concepts for the proposed modifications for the use of nutrient agar, stainless steel, and the target spore counts were previously identified and evaluated by other scientists (2–6; Rodriquez, 2002, unpublished data. The spore enumeration and neutralization procedures are based on existing protocols developed and used by the OPP Microbiology Laboratory. Prior to initiation of this collaborative effort, AOAC INTERNATIONAL assembled a review panel, the AOAC Sporicidal Method Expert Review Panel (ERP), to evaluate the collaborative study protocol. The ERP, along with members of the AOAC Methods Committee on Microbiology and Extraneous Materials, were engaged early in the development of the study design to ensure that the format would be acceptable to the AOAC *Official Methods*SM process. Based on the nature of the proposed modifications, a 4 laboratory study was agreed upon by the Study Director, the ERP, and the AOAC Methods Committee.

Collaborative Study

Applicability

The modifications are applicable to liquid formulations when tested against *B. subtilis* on a hard surface (porcelain carrier). The test chemicals used in the efficacy component of the study represent 3 different chemical classes: sodium hypochlorite (bleach), glutaraldehyde, and a combination of peracetic acid and hydrogen peroxide. The suitability of the modifications for porous materials (i.e., silk and polyester carriers) and gaseous formulations will require additional collaborative studies. Also, modifications to the

C. sporogenes component of the method (e.g., finding a suitable replacement for egg meat medium) will be evaluated under a separate study protocol.

Collaborators and Quality Management

Collaborators were chosen from government and private laboratories. Four laboratories participated in the study. A vendor supplied inoculated carriers to 2 of the 4 laboratories. The vendor verified the spore titer before shipment. The preparedness of each laboratory was assessed by the Study Director and Quality Assurance personnel prior to initiation of the study to ensure compliance with a project-specific EPA Quality Assurance Project Plan.

The Study Director provided method protocols, data sheets, media preparation sheets, test chemicals, selected media and reagents, garden soil, and porcelain penicylinders. The level of quality assurance was consistent with EPA Good Laboratory Practices (FIFRA 40 CFR Part 60). The garden soil used in the study was analyzed for pH as well as, nutrient and micronutrient content, and each production lot of test chemical was subjected to chemical analysis to verify that the formulations were within the certified specifications. Each laboratory designated a technician team to conduct the study. Based on the Study Director's experience with qualitative, technique-sensitive, carrier-based tests such as Method **966.04**, the data can be variable and the variance associated with technicians is usually negligible compared to the other sources of test variability. Nevertheless, if the same technician team always conducted the testing, the technician effect would be minimized.

Study Design

The current and modified methods are presented in Table 1 and the 3 medium/carrier combinations evaluated in this study are identified. Finding a suitable replacement for SENB was prioritized. Testing of a SENB/stainless steel combination received low priority and was not considered in this study. Each laboratory was instructed to determine carrier counts and HCl resistance for each medium/carrier combination, and if the established requirements were met, the carriers were considered suitable for efficacy testing.

Table 1. Current method and proposed replacements for Method 966.04 evaluated in the collaborative study

	Carrier (penicylinder) type	
Sporulation medium	Current method: Porcelain (PC)	Modified method: Stainless steel (SS)
Current method		
Soil extract nutrient broth (SENB)	SENB/PC	SENB/SS (not studied)
Modified method		
Amended nutrient agar (NA)	NA/PC	NA/SS

In addition, 2 laboratories (1 and 2) conducted a new procedure to evaluate the effectiveness of the prescribed neutralizers. Two laboratories (2 and 3) enumerated viable spores from individual carriers following exposure to the 3 low chemical treatments when tested with Method **966.04** (*see Quantitative Efficacy*). Two laboratories (2 and 3) performed a wash-off study to determine the impact of the carrier type on adherence properties of spores to the carriers using solutions of saline and saline + polysorbate 80. The various activities and distribution of the laboratory responsibilities are listed in Table 2. The procedures for each study are provided in this report.

Due to the instability of the test chemicals (sporicidal products), it was necessary for each laboratory to prepare test formulations on-site; thus, a blind assessment of each chemical treatment was not possible. To minimize any potential bias by the analysts, the order of testing chemicals against the medium/carrier combinations for each laboratory was randomized. The Study Director provided a randomized test plan to each laboratory. In order to have a balanced design that was conducive to a straight-forward statistical analysis,

Table 2. Collaborative activities and responsibilities of the laboratories

Activity	Lab No. 1	Lab No. 2	Lab No. 3	Lab No. 4
Produce spores with SENB and NA	Conducted by vendor[a]	Conducted by vendor	Yes	Yes
Inoculate carriers (PC and SS)	Conducted by vendor	Conducted by vendor	Yes	Yes
Conduct spore enumeration	Yes	Yes	Yes	Yes
Conduct HCl resistance tests	Yes	Yes	Yes	Yes
Conduct neutralization tests	Yes	Yes	No	No
Conduct efficacy tests	Yes	Yes	Yes	Yes
Conduct quantitative efficacy	No	Yes	Yes	No
Conduct wash-off in saline	No	Yes	Yes	No

[a] Conducted per instructions provided by the Study Director. Vendor was Presque Isle Cultures (Erie, PA).

each laboratory performed the same number of efficacy tests per day.

Due to the cost and the labor-intensive nature of conducting Method **966.04**, 30 carrier efficacy tests rather than 60 carrier tests were conducted. Each medium/carrier combination was tested against one chemical type (both high and low treatments) on the same day (a total of six 30 carrier tests per day). Two of the 4 laboratories used inoculated carriers prepared by a vendor (Presque Isle Cultures, Erie, PA); 2 laboratories prepared and inoculated their own carriers. The vendor, per instructions provided by the Study Director, followed the same protocol for the generation of the spore suspensions and inoculation of carriers as was provided to the collaborators.

Test Chemicals

Test chemicals are 3 commercially available antimicrobial products; the active ingredient(s) for each are (*1*) a combination of 0.08% peracetic acid/1.0% hydrogen peroxide; (*2*) 2.6% glutaraldehyde; and (*3*) 6.0% sodium hypochlorite (household bleach) without whiteners. For the purpose of this study, the test chemicals were used as experimental components only and were not tested to support or verify product label claims. Each chemical was tested as "high" (efficacious) and "low" (nonefficacious) to provide a range of efficacy, i.e., the high treatments were designed to generate passing results (0/30 positive carriers) and low treatments were designed to generate failing results (≥1/30 positive carriers). The test conditions used to generate the range are shown in Table 3. Thus, each medium/carrier combination was tested against a total of 6 chemical treatments.

Handling and Preparation of Test Chemicals

Test chemicals and the *Material Safety Data Sheets* for each were provided to each collaborator by the Study Director. Multiple lots of the chemicals were allowed into the study. Formulation chemistry analysis was performed on each lot to confirm the percent active ingredient(s). Four lots of the bleach product were available for testing; the lots ranged from 7.0 to 6.1% sodium hypochlorite. Three lots of the peracetic acid/hydrogen peroxide product were available for testing; the lots ranged from 0.08 to 0.75% and 1.0 to 0.97% for peracetic acid and hydrogen peroxide, respectively. Three lots of the glutaraldehyde product were used in the study; the lots ranged from 2.61 to 2.55% glutaraldehyde. Test parameters for each test chemical, describing the conditions for testing (e.g., dilution, neutralizer, contact time, temperature, etc.), were provided to each laboratory.

Table 3. Test conditions used to establish range of efficacy

	Test conditions	
Test chemical	High treatment	Low treatment
Peracetic acid/hydrogen peroxide	30 min exposure	5 min exposure
Glutaraldehyde	8 h exposure	1 h exposure
Sodium hypochlorite (bleach)	pH Adjusted to ca 7.0; 60 min exposure	pH Unadjusted (ca 10.0); 10 min exposure

Media and Reagents

(**a**) *Culture media.*—(*1*) *Nutrient broth*.—For use in preparing nutrient agar. Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (anatone, peptic hydrolysate of pork tissues, manufactured by American Laboratories, Inc., Omaha, NE) to ca 1 L water. Boil mixture for 20 min with constant stirring. Readjust volume to 1 L with water and allow cooling to around 50°C. Adjust pH to 6.8 ± 0.2 with 1 N HCl or 1 N NaOH, if necessary. Filter through paper (e.g., Whatman No. 4). Dispense 10 mL portions into 20 × 150 mm culture tubes or 20 mL portions into 25 × 150 mm culture tubes. Dehydrated nutrient broth may be substituted; prepare according to manufacturer's instructions. (*2*) *Nutrient agar*.—For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized nutrient broth. Boil mixture until agar is dissolved. Adjust pH to 7.2 ± 0.2 if necessary. Dispense 5 mL portions into 16 × 100 mm screw-cap tubes. Larger tubes may be used as well. Autoclave for 20 min at 121°C. Remove from autoclave and slant tubes to form agar slopes. (*3*) *Nutrient agar with 5 μg/mL $MnSO_4$–H_2O (amended nutrient agar)*.—For spore production. Suspend 11.5 g nutrient agar in 495 mL water, and add 5 mL 500 ppm $MnSO_4$–H_2O. Dissolve by boiling. Adjust pH to 6.8 ± 0.2 if necessary. Autoclave for 15 min at 121°C. Pour agar into plates. (*4*) *SENB*.—Extract 1 lb (454 g) garden soil (from Odenton, MD, collected and supplied by Study Director) in 1 L H_2O, filter several times through Schleicher & Schuell (Keene, NH) No. 588 paper, and dilute to volume (pH should be ≥5.2). Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone. Boil 20 min, dilute to volume, adjust with 1 M NaOH to pH 6.9 ± 0.2, and filter through paper. Dispense in 10 mL portions into 25 × 150 mm tubes, and autoclave 20 min at 121°C. (*5*) *Trypticase soy agar (TSA)*.—Suspend 40 g dehydrated TSA in 1 L water and heat gently while stirring. Boil 1 min or until completely dissolved. Adjust pH to 7.3 ± 0.2. Autoclave 15 min at 121°C. Pour agar into plates. (*6*) *Fluid thioglycollate medium (FTM)*.—Suspend 29.5 g dehydrated FTM in 1 L water. Heat to boiling to dissolve completely. Adjust pH to 7.1 ± 0.2 if necessary. Dispense 10 mL portions into 20 × 150 mm culture tubes and autoclave for 15 min at 121°C. Store at room temperature. Protect from light. *Note*: If after autoclaving, the aerated portion of media consumes more than one-third of tube, media must be reboiled by placing tubes in beaker of boiling water. Media can only be reboiled once. (*7*) *FTM with 1 M NaOH (modified FTM)*.—For subculturing spores exposed to 2.5 M HCl. Suspend 29.5 g FTM in 1 L water. Heat boiling to dissolve completely. Cool and adjust pH to 7.1 ± 0.2 if necessary. Add 20 mL 1 M NaOH and mix well.

Check final pH and record. No set pH for final product. Dispense 10 mL into 20 × 150 mm culture tubes and autoclave for 15 min at 121°C. Store at room temperature. Protect from light. *Note*: If after autoclaving, the aerated portion of media consumes more than one-third of tube, media must be reboiled by placing tubes in beaker of boiling water. Media can only be reboiled once. (*8*) *Letheen broth*.—Suspend 25.7 g letheen broth in 1 L water. Heat to boiling to dissolve medium completely. Adjust pH to 7.0 ± 0.2. Dispense 10 mL portions into 20 × 150 mm culture tubes. Autoclave 15 min at 121°C. (*9*) *Modified letheen broth with 0.1% (w/v) sodium thiosulfate*.—Suspend 25.7 g letheen broth in 1 L water and add 1.0 g sodium thiosulfate. Heat to boiling to dissolve completely. Adjust pH to 7.0 ± 0.2. Dispense into tubes. Autoclave 15 min at 121°C.

(**b**) *Manganese sulfate monohydrate*.—500 ppm. Add 0.25 g manganese sulfate to 500 mL water. Filter-sterilize for use.

(**c**) *Dilute hydrochloric acid*.—2.5 M. Use to determine resistance of dried spores. Standardize and adjust to 2.5 M as in Method **936.15**.

(**d**) *Water*.—Use sterile reagent grade water or sterile high performance liquid chromatography (HPLC) grade water or tap water where specified. Reagent grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent grade water when used in the proper arrangement. *See Standard Methods for the Examination of Water and Wastewater* for details on reagent grade water.

(**e**) *Sterile 5% acetic acid*.—Pour ca 500 mL 5% acetic acid into sterile disposable 0.22 µm filter unit in the biosafety cabinet (BSC). Filter-sterilize, and cap bottle before removing from BSC.

(**f**) *0.85% saline*.—Add 8.5 g NaCl to 1 L volumetric flask partially filled with water. Add water to bring to volume. Dissolve by stirring. Dispense into media bottles. Autoclave for 20 min at 121°C.

(**g**) *Saline + polysorbate 80*.—Measure 999 mL sterile 0.85% saline. Add 1 mL polysorbate 80. Mix thoroughly. Filter-sterilize, using 0.22 µm filter. Dispense into media bottles.

(**h**) *Test organism.—Bacillus subtilis* (ATCC No. 19659) obtained directly from a reputable supplier (e.g., ATCC).

(**i**) *Test chemicals*.—(*1*) *Bleach-unadjusted pH (pH about 10.0), 1:10 overall dilution (about 6000 ppm)*.—One part bleach, 9 parts sterile HPLC grade water. Bleach-unadjusted pH must be tested within ca 90 min after preparation. Used for the low treatment. (*2*) *Bleach-adjusted pH (pH 7.0 ± 0.5), 1:10 overall dilution (about 6000 ppm)*.—One part bleach, 0.6 parts 5% acetic acid, 8.4 parts sterile HPLC grade water. Bleach-adjusted pH must be tested within ca 90 min after preparation. Used for the high treatment. (*3*) *Peracetic acid/hydrogen peroxide product*.—Ready-to-use product and no dilution required, must initiate testing within ca 3 h after dispensing. (*4*) *2.6% glutaraldehyde product*.—Activated according to directions; product has a 14 day shelf life after activation. Testing was initiated within 3–5 days after activation.

(**j**) *Garden soil.*—From Odenton, MD, collected and supplied by Study Director.

Apparatus

(**a**) *Carriers*.—Penicylinders, porcelain, 8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm length (available from CeramTec Ceramic, Laurens, SC, USA, www.ceramtec.com, Cat. No. LE15819). Penicylinders, polished stainless steel, 8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm length; type 304 stainless steel, SS 18-8 (Fisher Scientific Cat. No. 07-907-5, Pittsburgh, PA).

(**b**) *Inoculated carriers*.—Inoculated porcelain and stainless steel carriers from Presque Isle Cultures (Erie, PA). *Note*: The same production lot of inoculated carriers was tested by 2 laboratories.

(**c**) *Glassware*.—For disinfectant, 25 × 150 mm or 25 × 100 mm culture tubes (Bellco Glass Inc., Vineland, NJ); reusable or disposable 20 × 150 mm (for cultures/subcultures); 16 × 100 mm screw-cap tubes for stock cultures. Cap with closures before sterilizing. Sterilize all glassware 2 h in hot air oven at 180°C or steam-sterilize for a minimum of 20 min at 121°C with drying cycle.

(**d**) *Sterile centrifuge tubes*.—Polypropylene, 50 mL conical tubes with conical bottoms (Fisher Scientific; Corning, Inc., New York, NY), Cat. No. 05-538-53D, or equivalent.

(**e**) *Chiller unit.*—Constant temperature for test chemical, capable of maintaining 20 ± 1°C temperature.

(**f**) *Water bath.*—For heat shock and for tests conducted at 25 ± 1°C, constant temperature, capable of maintaining 80 ± 2°C temperatures.

(**g**) *Petri dishes*.—Plastic (sterile), Fisher Scientific Cat. No. 08-757-103-C; VWR (West Chester, PA), Cat. No. 25373-143, or equivalent.

(**h**) *Filters*.—Schleicher & Schuell (S&S) filters (No. 588), 32 cm pleated: VWR Cat. No. 14224-414; used for preparation of SENB.

(**i**) *Filter paper*.—Whatman No. 2, Fisher Scientific Cat. No. 09-8100; VWR Cat. No. 28455-085, or equivalent; placed in Petri dishes for storing carriers.

(**j**) *Analytical filter unit*.—Nalgene 150 mL filter unit, 0.2 µm filter.

(**k**) *Test tube racks*.—Any convenient style.

(**l**) *Inoculating loop*.—Any convenient inoculation/transfer loop for culture transfer.

(**m**) *Wire hook*.—For carrier transfer. Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gage. Have other end in suitable holder.

(**n**) *Tissue homogenizer*.—Thomas Scientific (Swedesboro, NJ) 3431-E20 size B, or equivalent, used for preparation of SENB.

(**o**) *Centrifuge*.—Eppendorf 5804 R, or equivalent.

(**p**) *Sonicator*.—Branson (Danbury, CT) Model 1510, or equivalent.

(**q**) *Orbital shaker*.—VWR, Model DS-500, or equivalent.

(**r**) *Vacuum desiccator*.—For carrier storage. With adequate gauge for measuring 27 in. (69 cm) Hg and fresh desiccant.

(**s**) *Certified BSC (Class I or II)*.—Recommended for use to maintain aseptic work environment.

(**t**) *Certified timer*.—For managing timed activities. Calibration of the timers, against a National Institute of Science and Technology (NIST; Gaithersburg, MD) traceable standard, is recommended.

(**u**) *Glass wool*.—Fisher Scientific Cat. No. 11-390, used for preparation of SENB.

(**v**) *Inoculating loop*.—Make 4 mm id single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA). Fit other end in suitable holder. Bend loop at 30° angle with stem. Volumetric transfer devices may be used instead of transfer loops.

Procedure

(**a**) *Culture initiation*.—Reconstitute lyophilized *B. subtilis* culture with nutrient broth. Using several drops of the suspension, inoculate a second tube of nutrient broth. Incubate culture 24 ± 2 h at 37 ± 1°C. Use the 24 ± 2 h broth culture to initiate stock cultures (working slants). Streak inoculate a set (e.g., 6) nutrient agar slopes and incubate 24 ± 2 h at 37 ± 1°C. Concurrently, perform purity and identification confirmation testing (e.g., colony morphology on TSA, Gram stain, or other identification systems) on the initial 24 ± 2 h broth culture. After incubation, store at 2–5°C. Maintain stock culture on nutrient agar slants by monthly (30 ± 2 days) transfers. Every 30 ± 2 days, inoculate a new set of stock culture tubes from a current stock culture tube. Perform monthly quality control of stock cultures just prior to or concurrently with monthly stock culture transfers. Repeat cycle for 1 year, and then initiate a new culture.

(**b**) *Production of B. subtilis spore suspension from SENB*.—Laboratories prepared and harvested a test culture of *B. subtilis* according to Method **966.04**. The spores were used to inoculate porcelain carriers only. Extract 1 lb (454 g) garden soil (from Odenton, MD, collected and supplied by Study Director) in 1 L H_2O, filter several times through S&S No. 588 paper, and dilute to volume (pH should be ≥5.2). Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone. Boil 20 min, dilute to volume, adjust with 1 M NaOH to pH 6.9 ± 0.2, and filter through paper. Dispense in 10 mL portions into 25 × 150 mm tubes, and autoclave 20 min at 121°C. Inoculate each tube of SENB with several loopfuls of growth from the nutrient agar slants. Incubate tubes in slanted position for 72 ± 2 h at 37 ± 1°C.

Note: To enhance the level of spore production, use the following guidance (not part of the current method): Air-dry and sift soil to break large clumps before preparing the extract. Soak soil overnight in 1 L water. Filter entire volume. The filters clog easily; thus, frequently change filters. Allow filtered soil extract to settle overnight. Decant supernatant and discard particulate matter before use. Yield for soil extract will be approximately 600 mL. Also, after autoclaving the SENB tubes, allow them to settle overnight. If SENB tubes show settlement in bottom of tubes, remove media without disturbing pellet and transfer medium to another set of sterile tubes (adjust final volume in tubes to 10 mL). Alternatively, SENB may be autoclaved in flask, allowed to settle overnight, and then dispensed into test tubes. Avoid disturbing settlement during transfer.

(**c**) *Production of B. subtilis spore suspension from amended nutrient agar*.—Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of nutrient broth and incubate tubes on an orbital shaker at ca 150 rpm for 24 ± 2 h at 37 ± 1°C, repeat for a total of two 24 ± 2 h subcultures. Use this culture to inoculate amended nutrient agar plates. Inoculate each plate with 500 µL broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12–14 days at 37 ± 1°C. After incubation, harvest the spores by adding 10 mL cold sterile water to each plate. Using a spreader (e.g., bent glass rod), remove growth from plates and pipet suspensions into 50 mL sterile conical tubes (10 plates = 8 tubes, about 12 mL each). Centrifuge tubes at 5000 rpm for ca 10 min at room temperature. Remove and discard supernatant. Resuspend pellet in each tube with 20 mL cold sterile water and centrifuge at 5000 rpm for approximately 10 min. Remove and discard supernatant. Repeat twice. Resuspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2–5°C. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spores. Examine a minimum of 5 fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores vs vegetative cells should be at least 95%. Note debris if present. Spore suspension from multiple plates can be combined and re-aliquoted into tubes for uniformity. Before inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0×10^{-6} through 1.0×10^{-8}) using pour or spread plating on TSA plates. For pour plating, add molten TSA tempered to 45–55°C to each plate. Swirl pour plates to distribute spores evenly and allow agar to solidify. Invert plates and incubate for 24–48 h at 37 ± 1°C. Count colonies (by hand or with colony counter). Use dilutions yielding between 30 and 300 colony-forming units (CFU) per plate (target counts) for enumeration; however, record all counts less than 30. Report plates with colony counts over 300 as too numerous to count (TNTC). *Note*: When harvested and processed, 10 plates of amended nutrient agar should provide 80–100 mL concentrated spore suspension (ca 10^9 CFU/mL). Diluting the suspension before carrier inoculation will be necessary; a titer of 1.0×10^8–5.0×10^8 CFU/mL should be adequate to achieve the target carrier count.

(**d**) *Preparation of porcelain carriers*.—Before use, examine porcelain carriers individually and discard those with scratches, nicks, spurs, or discolorations. Rinse unused

carriers gently in water 3 times to remove loose material, and drain. Place rinsed carriers into Petri dishes matted with 2 layers of filter paper in groups of 15 carriers per Petri dish. Sterilize 20 min at 121°C. Cool and store at room temperature. *Note*: Handle porcelain carriers with care when placing in Petri dishes. Minimize carrier movement and avoid excessive contact between carriers that might result in chips and cracks. Wash carriers with Triton X-100, and rinse with water 4 times for reuse.

(**e**) *Preparation of stainless steel carriers.*—Physical screening: examine steel carriers individually and discard those with scratches or nicks. Soak overnight (ca 12 h) in 1 N NaOH and rinse 3–4 times with tap water. Collect portion of last rinse water and add 2–3 drops 1% phenolphthalein. If any NaOH remains, phenolphthalein turns pink, indicating need for additional rinsing. Continue to rinse carriers until addition of phenolphthalein does not produce a color change. Rinse twice more with water (reagent grade). Place rinsed carriers into Petri dishes matted with 2 layers of filter paper in groups of 15 carriers per Petri dish. Sterilize 20 min at 121°C. Store sterile carriers at room temperature for up to 3 months. After 3 months, reclean and sterilize carriers before use.

(**f**) *Inoculation of porcelain carriers with spore suspensions prepared using SENB.*—Pour contents of 1 tube of a 72 ± 2 h SENB culture into sterile tissue grinder (Thomas Tissue Homogenizer) and macerate pellicle, several (15–20) strokes for each tube. Filter through sterile funnel containing moist glass wool into sterile container. Repeat for remaining tubes. Pool spore filtrates into separate holding container. Pipet 10 mL volumes into sterile 25 × 150 mm test tubes agitating holding container between each 10 mL transfer to maintain spores in suspension. Add 10 sterile penicylinders to each tube containing 10 mL spore filtrate and let stand 10–15 min. Carriers should remain completely immersed in inoculum during exposure period. Remove carriers with sterile hook and place upright in sterile, double matted (2 layers of filter paper) Petri dishes, no more than 30 carriers per Petri dish. Allow to air-dry in BSC for ca 30 min. Place Petri dishes containing contaminated carriers in vacuum desiccator containing $CaCl_2$ and draw vacuum of 69 cm (27 in.) Hg. Maintain carriers under vacuum 24 ± 2 h before use. For long-term storage, spore carriers must be maintained under vacuum and can be used for up to 3 months. Carriers older than 3 months should be requalified by both standard HCl test and spore quantitation before use.

(**g**) *Inoculation of porcelain and stainless steel carriers with spore suspension prepared using amended nutrient agar.*—Add 10 sterile carriers to each tube containing 10 mL spore suspension, agitate slightly, and let stand 10–15 min. Remove each carrier with sterile hook and place upright in sterile Petri dish lined with 2 sheets of filter paper, no more than 30 carriers per Petri dish. Air-dry in BSC for ca 30 ± 2 min. Place Petri dishes containing inoculated carriers in vacuum desiccator containing $CaCl_2$ and draw vacuum of 69 cm (27 in.) Hg for 20 ± 2 min. Dry carriers under vacuum of 69 cm (27 in.) Hg for a minimum 24 ± 2 h before use in HCl resistance, efficacy testing, or carrier counts. Spore carriers should be maintained under vacuum. *Note*: Ten plates of amended nutrient agar should provide up to 100 mL spore suspension which can be diluted with sterile water to provide spores in the range of 1.0×10^8–5.0×10^8 CFU/mL.

(**h**) *Spore enumeration (carrier counts).*—Before use, determine the carrier counts for each preparation of carriers. Assay 5 randomly selected carriers per preparation. Place each inoculated carrier into a 50 mL plastic, polypropylene conical centrifuge tube containing 10 mL sterile water. Sonicate carriers for 5 min ± 30 s. *Note*: For sonication, place tubes into an appropriately sized beaker with tap water to the level of sterile water in the tubes. Place beaker in sonicator so that water level in the beaker is even with water level fill line on sonicator tank. Fill tank with tap water to water level fill line. Suspend beaker in sonicator tank so that it does not touch bottom of tank and all 3 water levels (inside test tubes, inside beaker, and sonicator tank) are the same. After sonication, mix tubes in a Vortex mixer for 2 min ± 5 s. Dilute spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile water. Dilute spore suspensions out to 1.0×10^{-5} and plate dilutions 1.0×10^{-2} through 1.0×10^{-5}. Plate each dilution in duplicate using pour or spread plating with TSA and determine titer. *Note*: The mean carrier counts for each set of inoculated carriers, including those supplied by the vendor, must be at an acceptable level (a minimum of 1.0×10^5 spores/carrier and a maximum of approximately 1.0×10^6 spores/carrier) to be used in HCl resistance and efficacy testing. Laboratories were instructed not to use carriers if average counts fell below the minimum or above the approximate maximum.

(**i**) *HCl resistance*.—Equilibrate water bath to 20 ± 1°C. Pipet 10 mL of 2.5 M HCl into two 25 × 100 mm tubes and place into water bath. Allow at least 30 min to reach temperature equilibrium. Start timer and rapidly transfer 4 inoculated penicylinders into an acid tube (2.5 M HCl) with flamed hooks or forceps. Do not allow carriers or transfer device to contact inside of wall of acid tube. Transfer individual carriers after 2, 5, 10, and 20 min of HCl exposure to a separate tube of modified FTM. Rotate each tube vigorously by hand for ca 20 s and then transfer carrier to a second tube of modified FTM. For viability control, place 1 unexposed inoculated carrier in a separate tube of modified FTM. For media sterility, use 1 tube of modified FTM. Incubate all test and control tubes for 21 days at 37 ± 1°C. Record results as growth (+) or no growth (–) at each time period. Spores should resist HCl for ≥2 min to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described.

(**j**) *Efficacy test.*—One chemical type was tested per test day. Bleach and peracetic acid/hydrogen peroxide were tested at 20 ± 1°C. Glutaraldehyde was tested at 25 ± 1°C. For the bleach treatments, letheen broth with 0.1% sodium thiosulfate was used as the neutralizer (primary tube) and FTM was the subculture medium (secondary tube). For the peracetic acid/hydrogen peroxide and glutaraldehyde treatments, FTM was used as the neutralizer and subculture medium. For a 30 carrier test, place 10 mL product at dilution recommended

for use or under investigation into each of six 25 × 150 mm or 25 × 100 mm tubes (medication tubes). Place tubes in 20 ± 1°C water bath and let equilibrate to temperature. Using a sterile hook or forceps, transfer inoculated carriers sequentially at 2 min intervals in groups of 5 from Petri dish to medication tubes containing sporicidal agent. Use a certified timer to monitor time. Flame hook and allow cooling after each transfer. When lowering carriers into medication tube, neither carriers nor wire hook may touch sides of tubes. If interior sides are touched, note tube number. Do not count carrier set if any carrier from that group of 5 yields a positive result. Inadvertent contamination of this type may result in retesting. Carriers must be deposited into medication tubes within ±5 s of the prescribed drop time. Return tubes to water bath immediately after adding carriers. After contact period has been achieved, transfer carriers in same sequential timed fashion into primary subculture tubes containing appropriate neutralizer (10 mL in 20 × 150 mm tubes). Remove the carriers one at a time from the sporicidal agent medication tube with sterile hook, tap against interior side of tube to remove excess sporicidal agent, and transfer into neutralizer tube (primary tube). All 5 carriers must be transferred during each 2 min interval. Flame hook between each carrier transfer. Move remaining carriers into their corresponding neutralizer tubes at appropriate time. Carriers may touch interior sides of neutralizer tube during transfer, but contact should be minimized. After each carrier is deposited, recap neutralizer tube and gently shake to facilitate adequate mixing and efficient neutralization. Within 1 h from when last carrier was deposited into primaries, transfer carriers using sterile wire hook to second subculture tube (secondary tube) containing 10 mL appropriate recovery medium, 1 carrier per tube. Move carriers in order, but movements do not have to be timed. Gently shake entire rack of secondary tubes after all carriers have been transferred. Incubate primary (neutralizer) and secondary subculture tubes for 21 days at 37 ± 1°C. Report results as growth (+) or no growth (–). A positive result is one in which medium appears turbid. A negative result is one in which medium appears clear. Shake each tube before recording results to determine presence or absence of growth/turbidity. Primary and secondary subculture tubes for each carrier represent a carrier set. A positive result in either primary or secondary subculture tube is considered a positive result for the carrier set. If no growth occurs after 21 days, heat shock all test and control tubes for approximately 20 min at 80 ± 2°C and re-incubate for 72 ± 2 h at 37 ± 1°C.

Media sterility controls and system controls (check for aseptic technique during carrier transfer process) are recommended. For media controls, incubate 1–3 unopened subculture medium tubes with the test sample tubes for 21 days at 37 ± 1°C. For system controls, use sterile needle hook to transfer 3 sterile carriers into a tube of test chemical. Transfer system control carriers to neutralizer medium as follows: At start of sample test (prior to first tube), transfer 1 sterile carrier to tube of neutralizer medium. After one-half of test carriers have been transferred to neutralizer tubes, transfer a second sterile carrier to tube of neutralizer medium. After all test carriers (last tube) have been transferred to neutralizer tubes, transfer third sterile carrier to tube of neutralizer medium. Transfer system control carriers to secondary subculture medium as follows: Immediately before initiating transfer of test carriers into secondary subculture medium tubes, transfer first system control sterile carrier from neutralizer medium to tube of subculture medium. After one-half of test carriers have been transferred to secondary subculture medium tubes, transfer second system control sterile carrier to tube of subculture medium. After all test carriers have been transferred to secondary subculture medium tubes, transfer third system control sterile carrier to tube of subculture medium. For each test, include a positive carrier control by placing 1 inoculated carrier into tube of secondary subculture medium. Incubate controls and test sample tubes together for 21 days at 37 ± 1°C.

Perform identification confirmation on a minimum of 3 positive carrier sets per test, if available, using Gram stain and/or plating on TSA. Additional confirmation may be performed using VITEK, API analysis, or comparable method. If fewer than 3 positive carrier sets, confirm growth from each positive carrier set. If both tubes are positive in a carrier set, select only 1 tube for confirmatory testing. For test with 20 or more positive carrier sets, confirm at least 20% by Gram stain.

(**k**) *Neutralization confirmation procedure.*—The neutralization (inactivation) of active ingredients is one of the most important steps in efficacy testing of antimicrobial products. Neutralization is used to stop the activity of the product ingredients, a process essential to measuring effectiveness at a desired contact time. In addition, the neutralizer itself or in combination with the recovery medium must not exhibit bacteriostatic activity against the test microbe. Neutralization can be accomplished by various means (e.g., dilution, use of specific neutralizing media, physical and chemical effects).

The neutralizers for the products tested in the collaborative study were previously determined to be effective by the Study Director using the procedure below; however, for the purpose of the collaborative study, 2 laboratories independently performed the procedure to ensure its clarity and usefulness. This procedure simulates the conditions of the efficacy test, except that sterile carriers were used instead of inoculated carriers, and provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer/disinfectant interactions. The test chemicals and parameters were (*1*) bleach adjusted pH (about 6000 ppm with 60 min exposure); (*2*) peracetic acid/hydrogen peroxide (30 min exposure); and (*3*) 2.6% glutaraldehyde (60 min exposure). Only the porcelain carrier type was used in this study. The selection of test chemicals and associated parameters were used to simulate more challenging scenarios for chemical inactivation.

Produce a spore preparation according to procedure for amended nutrient agar. Harvest growth from plates (e.g., 5 plates) per the method, except resuspend pellet after final

centrifugation step in approximately 100 mL aqueous (40%) ethanol. Store the spore suspension at 2–5°C. Determine spore count by serial dilution and plating (pour or spread) on TSA. Desirable target of the initial working suspension is 1.0×10^8 to 1.0×10^9 CFU/mL. The suspension may require adjustment to reach target titer. Prepare serial 10-fold dilutions of the inoculum in sterile water out to 10^{-8}. Use 10^{-6}, 10^{-7}, and 10^{-8} dilutions to inoculate the neutralizer and subculture media tubes. The target number of spores to be delivered per tube in this assay is 5–100 spores/tube. Determine spore titer by plating (pour or spread) each of 3 dilutions in duplicate on TSA.

Use a set of 5 sterile carriers (only 3 to be used in the assay). Within 5 s, place a set of 5 carriers into a glass tube (25 × 150 mm or 25 × 100 mm) containing test chemical; transfer carriers according to Method **966.04**. Allow carriers to remain in test chemical per the specified contact time and temperature. After the contact time is complete, aseptically transfer 3 of the 5 carriers individually into tubes containing the neutralizer per Method **966.04**. This set of tubes is the neutralizer/primary subculture treatment. After transfering the last carrier into neutralizer tube, transfer each carrier, in sequence, into a tube containing secondary subculture medium. This portion of assay is not timed, but should be made as soon as possible. This set is the secondary subculture treatment. After carrier transfer, inoculate each tube (neutralizer/primary and secondary subculture treatment tubes) with 1 mL of each of 3 inoculum dilutions (10^{-6}, 10^{-7}, and 10^{-8}). For controls, use 3 fresh, unexposed tubes of neutralizer and 3 tubes of the secondary subculture medium; also inoculate each control tube with 1 mL of each of 3 inoculum dilutions. Include 1 uninoculated tube of neutralizer and secondary subculture media to serve as sterility controls. *See* Table 4 for tube inoculation scheme. Incubate all tubes 5–7 days at 37 ± 1°C. Record results as growth (+) or no growth (–). *Note*: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

Confirm a minimum of one positive tube per treatment and control (if available) using Gram staining and colony morphology on TSA. For each treatment and control group, conduct confirmation testing on growth from tube with fewest spores delivered. *B. subtilis* is a Gram-positive rod, and colonies on TSA are opaque, rough, dull, round, with irregular margins, and low convex. Growth in the inoculated controls verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *Note*: There may be cases when the neutralizer is significantly different from the secondary subculture media; in these cases, growth may not be comparable. The uninoculated control tubes are used to determine sterility and must show no growth for the test to be valid.

The occurrence of growth in the neutralizer/primary subculture and secondary subculture treatment tubes is used to assess the effectiveness of the neutralizer. No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For a neutralizer to be deemed effective, growth must occur in the secondary subculture treatment tubes which received lower levels of inoculum (e.g., 5–100 CFU/tube).

(**l**) *Quantitative efficacy*.—This study was conducted independently of the efficacy evaluation. For each low chemical treatment, 2 laboratories tested 5 carriers of each medium/carrier combination according to the randomization scheme provided by the Study Director. The low treatments were used to provide a level of detectable viable spores for comparative analysis.

Conduct efficacy tests (5 carriers) for each of 3 combinations of carriers according to Method **996.04**. Transfer carriers sequentially at 2 min intervals in groups of 5 from the Petri dish into test tubes containing sporicidal agent using a sterile hook. Immediately after placing carriers into medication tube, return to water bath held at 20 ± 1°C (or specified temperature). Carriers must be deposited into tube within ±5 s of prescribed drop time. Flame hook and allow cooling after each carrier transfer. *Note*: When lowering carriers into test tube, neither carriers nor wire hook may touch sides of tubes. If interior sides are touched, note tube number. Do not count carrier set if any carrier from that group of 5 yields a positive result. Inadvertent contamination of this type may result in retesting.

After prescribed contact period, transfer carriers in same timed fashion into tubes containing sterile water (10 mL in 50 mL plastic conical bottom centrifuge tube). Transfer all

Table 4. Neutralization confirmation procedure: inoculating treatment and control tubes with diluted spore suspension[a]

Neutralizer–primary subculture treatment	Secondary subculture treatment (with carrier)	Neutralizer–primary inoculated control	Secondary subculture inoculated control
1 mL of 10^{-6} → Tube 1	1 mL of 10^{-6} → Tube 1	1 mL of 10^{-6} → Tube 1	1 mL of 10^{-6} → Tube 1
1 mL of 10^{-7} → Tube 2	1 mL of 10^{-7} → Tube 2	1 mL of 10^{-7} → Tube 2	1 mL of 10^{-7} → Tube 2
1 mL of 10^{-8} → Tube 3	1 mL of 10^{-8} → Tube 3	1 mL of 10^{-8} → Tube 3	1 mL of 10^{-8} → Tube 3

[a] 1.0×10^{-6} through 1.0×10^{-8} based on starting suspension of approximately 10^8 spores/mL.

5 carriers during each 2 min interval. Flame hook between each carrier transfer. Place tubes with carriers into appropriately sized beaker and fill beaker with tap water to level of sterile water in tubes. Place beaker in sonicator so water level in beaker is even with water level fill line. Water in tank should never fall below 1 in. from top of tank. Place beaker in sonicator tank so that it is not touching bottom and all 3 water levels (inside test tubes, inside beaker, and sonicator tank) are the same. Sonicate samples 5 min ± 30 s. After sonication, mix each tube in a Vortex mixer 2 min ± 5 s. Dilute spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile water. Dilute spore suspensions out to 1.0×10^{-4} and plate 1 mL of appropriate dilutions (1.0×10^{-1} to 1.0×10^{-4}). Plate each dilution using pour plate or spread plate techniques with TSA. Visually examine morphology of colonies to confirm identity of culture. Calculate mean number of viable spores per medium/carrier combination and the log reduction (LR) for each chemical.

(**m**) *Wash-off of spores in saline*.—Analyze a total of 18 carriers. In separate test tubes (25 × 100 mm), expose sets of inoculated carriers (1 per tube) to 2 saline treatments: (*1*) 10 mL 0.85% saline and (*2*) 10 mL 0.85% saline + polysorbate 80 (final concentration 0.01%). Test 1 carrier type at a time. Without disturbing tubes, allow contact time for 10 min ± 30 s at 20 ± 1°C. Track elapsed time with certified timer. Remove carriers in sequential order from control solutions and place each into 50 mL plastic conical bottom centrifuge tube containing 10 mL sterile water. Place all tubes (6 total tubes/carrier × medium combination) with carriers into appropriately sized beaker and fill beaker with tap water to level of sterile water in tubes. Place beaker in sonicator tank so that it is not touching bottom and all 3 water levels (inside test tubes, inside beaker, and sonicator tank) are the same. Sonicate samples 5 min ± 30 s. After sonication, mix each tube in a Vortex mixer for 2 min ± 5 s. Dilute spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile water. Dilute spore suspensions out to 1.0×10^{-5} and plate 1 mL of appropriate dilutions (1.0×10^{-2}–1.0×10^{-5}). Plate each dilution in duplicate using pour plate or spread plate techniques with TSA. Visually examine morphology of colonies to confirm identity of culture.

To determine wash-off, plate serial dilutions of 1 mL aliquot of liquid in initial treatment tubes (saline and saline + polysorbate). For each test tube (18 total), mix briefly in a Vortex mixer, remove 1 mL and serially dilute in 9 mL sterile water to 1.0×10^{-4}. Plate 1 mL of appropriate dilutions (1.0×10^{-1}–1.0×10^{-4}) in duplicate using pour plate or spread plate techniques with TSA. Visually examine morphology of colonies to confirm identity of culture.

Data Analysis and Statistical Methods

Statistical comparisons were made between the current method (SENB/PC) versus each of the modified methods (NA/PC and NA/SS) with respect to control carrier counts, the pass/fail HCl resistance result, the pass/fail efficacy result, the observed numbers of positives among treated carriers, and the percentage washed-off during a simulated disinfection step. The mean and reproducibility standard deviation (SD_R) were presented for the quantitative measurements. The mean and SD_R calculations were performed separately for each of the 3 medium/carrier combinations. By submitting the data to a random effects analysis of variance (ANOVA), it was possible to estimate the within-experiment variance, the between-laboratory variance, and SD_R. (*Note*: The SD_R is the typical difference, sign neglected, between the measurement for a single, randomly chosen carrier or sample and the mean across many independent experiments in many laboratories. A small value for the SD_R indicates that the medium/carrier method produces about the same measurement from laboratory to laboratory).

Quantitative aspects of AOAC Method **966.04** (SENB/PC) were compared with the same aspects for the modified tests (NA/PC and NA/SS). Two types of statistical tests were conducted: the conventional hypothesis test and the equivalence test. For comparing the difference between the AOAC and modified methods, the results include a *P*-value and a 90% confidence interval, which are the key summary statistics for the conventional hypothesis test. The 90% confidence interval is also the key summary statistic for the equivalence test.

Many published articles and books describe the conventional and equivalence testing approaches (8, 9). The problem of comparing means will be used to motivate the ideas, i.e., suppose the analysis is to compare the true, unknown mean for SENB/PC, denoted by μ_S, to the true, unknown mean for one of the modifications, denoted by μ_N, based on the difference, denoted by δ. Then $\delta = \mu_S - \mu_N$. Because the ideas discussed here are quite general, it is not necessary to specify the laboratory measurement to which the mean applies. The measurement could be the log-transformed spores per carrier or it could be the LR, to list 2 specific examples.

The conventional hypothesis testing approach is directed at using the observed data to show that $\delta \neq 0$. The problem is formulated in the context of hypotheses, specifically the null hypothesis: H_o: $\delta = 0$ and the alternative hypothesis: H_a: $\delta \neq 0$. A decision rule is devised by which one decides whether or not to reject H_o in favor of H_a, depending on the observed laboratory data. The decision rule is established before any data are collected. It can be visualized as a partitioning of the list of all possible laboratory data values into 2 exclusive, exhaustive subsets called the "acceptance region" and the "rejection region." If the data realized in the actual experiment fall within the rejection region, then one would reject H_o and conclude that the observed difference between means is statistically significantly different from zero.

The equivalence testing approach is directed at using the observed data to show that δ is negligibly small. The equivalence test depends on a quantitative definition of "statistically equivalent." To put this idea in a specific context, consider the carrier count data for which the key summary statistic is the mean log spores per carrier. Equivalence testing requires specification of a nonzero numerical value, δ_a, for δ such that any δ in the "equivalence zone," $-\delta_a < \delta < \delta_a$, is judged to be negligibly small for all practical purposes. If δ is outside that equivalence zone, the 2 methods are judged to be

nonequivalent. The choice of δ_a is subjective, dependent on expert judgment about practicality and importance. For the purpose of this presentation, the Study Director selected the value $\delta_a = 1.0$. The equivalence test is conducted by comparing the 90% confidence interval to the equivalence zone as follows: If the 90% confidence interval for δ is completely contained within the equivalence zone ($-1.0 < \delta < 1.0$), one can conclude that the methods are statistically equivalent at a 0.05 level. If the 90% confidence interval extends outside the equivalence zone, then one cannot conclude that the methods are statistically equivalent. Although it may seem paradoxical to use a 90% confidence interval as the basis for a 0.05 level equivalence test, it is a mathematically justified, valid method (8, 9).

HCl Resistance Test

The quantitative results for both the HCl resistance test and the efficacy test were recorded as binary pass/fail data, following established performance standards. (*1*) AOAC Method **966.04** calls for a minimum of 2 min resistance to 2.5 M HCl. This is a pass. The outcomes (pass or fail) will be compared for each medium/carrier combination. The basis of the comparison is the current method SENB/PC. The modified version(s) must exhibit a statistically equivalent pattern of outcomes. (*2*) EPA/FDA requires killing on all test carriers; thus no positive carrier sets present. This is a pass. To validate the modification(s), the modified versions must exhibit a pass/fail pattern that is statistically equivalent to the current method (SENB/PC).

Carrier Counts

For each of the 3 medium/carrier combinations, the viable spores on each of 5 control carriers were counted in each laboratory. The spores per carrier approximately followed a log normal distribution. To create a measurement that exhibited normality and homogeneous variance, statistical analyses were performed on the $\log_{10}$-transformed spores per carrier (10). To compare a new medium/carrier combination NA (either NA/PC or NA/SS) to the standard medium/carrier combination SENB/PC, the mean log spores per carrier was calculated for each combination of the 2 methods and the 4 laboratories. Then the means for the methods were compared using a paired *t*-test, where the means were paired by laboratory.

Efficacy

Pass/fail results.—Let S denote the current standard method (SENB/PC) and N denote a new modification (NA/PC or NA/SS). Each medium/carrier combination was tested once for each chemical treatment in each laboratory. The N and S test outcomes for each combination of sporicide treatment and laboratory were treated as paired for purposes of statistical analysis. For each pair of tests, it is of interest to determine the extent to which the 2 tests resulted in the same outcome, pass or fail. Such paired binary data are conventionally presented in the 2×2 table format of Table 5.

Outcome combinations for counts a and d are *concordant* pairs (pass for both methods or fail for both methods) and outcome combinations for counts b and c are *discordant* pairs (fail for one method and pass for the other). The true, unknown probabilities for the 4 cells in Table 5 are denoted by P_a, P_b, P_c, and P_d, respectively. The probability of a pass for Method N is $P_N = P_a + P_c$ and the probability of a pass for Method S is $P_S = P_a + P_b$. The true difference between pass probabilities, denoted by D, is $D = P_S - P_N = P_b - P_c$, the difference between the pass probabilities for the 2 discordant pairs. The estimate of D is $(b - c)/n$ (11). To test the null hypothesis that $D = 0$ (i.e., the 2 methods produce the same pass probabilities), the Tango score test version of McNemar's test was used (11–14). The 90% confidence interval for D was calculated using the Tango method for the difference between 2 proportions based on paired data (12–15).

Percentages of agreement were calculated as follows: (*1*) the overall percentage agreement between N and S based on the estimate $[(a + d)/n]\cdot 100\%$; (*2*) the percentage agreement of N with a passed S based on the estimate $[a/(a + b)]\cdot 100\%$; and (*3*) the percentage agreement of N with a failed S based on the estimate $[d/(c + d)]\cdot 100\%$.

LR results using the positive/negative (P/N) formula.—The LR measure of efficacy is the difference on the $\log_{10}$ scale between the number of spores per control carrier and the number of spores per treated carrier. Let the mean of the log spores per carrier be denoted by C for control carriers and by T for treated carriers. Let LR denote the log reduction value. Then $LR = C - T$. Note that because C and T are the means of logs, LR is a measurement on the $\log_{10}$ scale. In this investigation, the numbers of spores on control carriers were counted and C was easily calculated. Although the spores per treated carrier were not counted, it was possible to estimate T based on the number of positive carriers among the 30 treated carriers and to calculate LR using the P/N formula in Equation 1.

Consider a disinfectant test that uses M_c control carriers that are subjected to quantitative evaluation and M_t treated carriers that are subjected to qualitative evaluation. Viable spore counts per carrier are observed for each of the control carriers, resulting in counts of $c_1, \ldots, c_{Mc}$. Denote the mean of the $\log_{10}$ counts by C; that is, $C = [\log(c_1) + \ldots + \log(c_{Mc})]/M_c$.

Each of the treated carriers is observed to be either positive or negative, where a carrier is positive if and only if there is at

Table 5. Summary of paired binary (pass/fail) data; each entry (a, b, c, or d) is the number of pairs having that combination of N and S outcomes

	Test outcome for N		
Test outcome for S	Pass	Fail	Total
Pass	a	b	a + b
Fail	c	d	c + d
Total	a + c	b + d	n = a + b + c + d

least one viable spore on the carrier. Let G denote the observed number of *negative* carriers and A denote the adjusted fraction of treated carriers that are negative, $A = (G + ½)/(M_t + 1)$.

Let ln(·) denote the natural logarithm, which is sometimes denoted by $\log_e(\cdot)$. The P/N formula for converting the control and treatment observations into an LR is

$$\text{P/N formula: LR} = C - \log_{10}[-\ln(A)] \qquad (1)$$

The rationale for this formula will now be described. Suppose the carriers were prepared so that there were V viable spores per carrier. Suppose each of the V spores on a treated carrier has probability p of being killed by exposure to the disinfectant. If we assume that the spores live or die independently, the number of spores that survive the disinfectant will follow a binomial (V, p) probability distribution.

Let Y_i denote the positive/negative outcome for the ith treated carrier, $i = 1, 2, \ldots, M_t$; specifically, $Y_i = 0$ or 1, where $Y_i = 0$ if the carrier is negative and $Y_i = 1$ if the carrier is positive. Then the probability that the ith carrier is positive is $\Pr\{Y_i = 1\} = 1 - (1 - p)^V$. If V is large, as it will be in disinfectant testing where V is usually greater than 10^5, the expression $(1 - p)^V$ is closely approximated by e^{-Vp}.

If the treated carriers are statistically independent, then the number of positive carriers, $Y_1 + \ldots + Y_{Mt}$, will follow a binomial$(M_t, 1 - e^{-Vp})$ probability distribution. Therefore, the expected number of negative carriers is $M_t \cdot e^{-Vp}$. Setting the observed number of negative carriers equal to the expected number and solving for p, we find that $G = M_t \cdot e^{-Vp}$ if and only if

$$p = -\ln(G/M_t)/V \qquad (2)$$

Let LR_{true} denote the true, unknown log reduction value. The LR is related to the survival probability for individual spores by the equation: $LR_{true} = -\log_{10}(p)$. Let LR* denote a preliminary estimate of LR_{true}. Substituting for p in Equation 2, a plausible estimator is $LR^* = -\log_{10}(-\ln(G/M_t)/V)$ or

$$LR^* = \log_{10}(V) - \log_{10}(-\ln(G/M_t)) \qquad (3)$$

For a real disinfectant test, the viable spores per carrier will not be identical. The typical number of spores per carrier is estimated by the geometric mean of control carrier counts, 10^C. Therefore, it is reasonable to replace the $\log_{10}(V)$ term in Equation 3 with $\log_{10}(10^C) = C$, the mean of log counts for control carriers. In Equation 3, G/M_t is the observed fraction of treated carriers that are negative. If $G = 0$, Equation 3 is not calculable because ln(0) is not defined mathematically. DeVries and Hamilton (16) explored a variety of ways to adjust the fraction A so that it is never zero. They arrived at the recommendation to replace G/M_t with $A = (G + ½) / (M_t + 1)$. Although the DeVries and Hamilton work focused on confidence interval calculations, their recommended adjusted fraction A was adopted here for the P/N formula. These practical substitutions for the 2 components of Equation 3 produce the P/N formula of Equation 1.

To compare a new medium/carrier combination NA (either NA/PC or NA/SS) to the standard medium/carrier combination SENB/PC, the LR was calculated for each combination of the 2 methods and the 4 laboratories. Then the LR values for the methods were compared using a paired t-test, where the means are paired by the combination of laboratory and treatment. The test outcome was summarized by a 2-tailed P-value. The paired t-method was also used to find a 90% confidence interval for the mean difference of LR values, SENB/PC – NA.

Quantitative Efficacy

Two laboratories measured the number of viable spores per treated carriers following exposure to the low treatments. This allowed the direct calculation of the log reduction, LR = (mean of log spores per control carriers) – (mean of log spores per treated carrier). The LR values for SENB/PC and NA were paired on laboratory by sporicide combination, and the paired t-test was used to find the p-values and 90% confidence intervals for the mean differences, SENB/PC – NA/PC and SENB/PC – NA/SS. For each medium/carrier, SD_R was the square root of the residual mean squared error for a 1-way ANOVA, where the factor was treatment (2 levels).

Wash-Off in Saline

Three carriers of each medium/carrier combination were exposed to 2 saline treatments (0.85% saline and 0.85% saline + polysorbate 80). The saline + polysorbate 80 (final concentration 0.01%) was used to increase the surfactancy of the saline, thus simulating an actual product. The percent wash-off for each medium/carrier combination was calculated as follows:

$$\%\ \text{Wash-off} = \frac{100 \times \text{mean number of wash-off spores in medication tubes}}{\text{by the total}}$$

where the total is the mean number of spores in suspension following wash-off plus the mean number of spores from subsequently removed by vortexing the carrier.

The percent wash-off and total spores/carrier were calculated for each saline treatment for each medium carrier combination; comparisons were made to the current method (SENB/PC).

Because the percentage washed-off is small for NA/SS, the homogeneous variance and normality assumptions required for the ANOVA were violated and it was necessary to log-transform the percentages washed-off prior to statistical analysis. A one-factor random effects ANOVA, where laboratory was the random effect, was conducted for each medium/carrier. Paired t-tests were used to compare the log-transformed wash-off percentages for a new test system N to the standard test system S. The percentages were paired by the combination of laboratory and treatment, where treatment refers to saline or saline plus polysorbate 80. Diagnostic statistical evaluation during the analyses showed that the log-transformation produced data that did conform to the

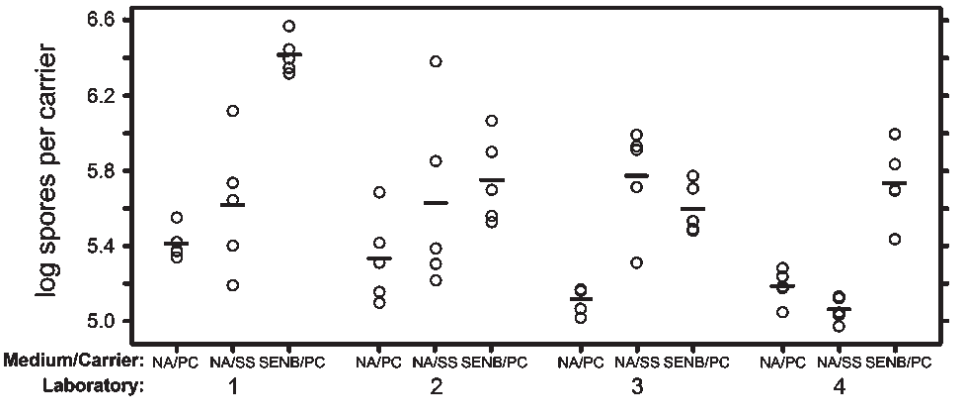

Figure 1. Log_{10} Spore counts per control carrier, arranged to show the variability within experiments, among methods, and among laboratories. Each point is one carrier. Points aligned vertically are from the same experiment. Each horizontal line is the mean of log spores per carrier for the experiment.

usual normality assumptions. The log-transformed total spores per carrier were analyzed by the same methods.

The antilog of the mean log percentage is the geometric mean of the percentage washed off. The antilog of the difference between the means of log-transformed percentages is the ratio of geometric means. The results for wash-off are presented in terms of geometric means and ratios of geometric means, although the statistical analyses were conducted on the log scale. The delta method was used to calculate the SD_R that is applicable to the percentage scale (11).

Results and Discussion

Carrier Counts

Attaining a target number of spores per carrier is desirable for standardizing Method **966.04** and increasing the method's reproducibility and quality of the data. To assess the feasibility of the proposed modification to establish a target minimum titer of 5 logs (1.0×10^5) of *B. subtilis* spores per carrier, each of the 4 laboratories independently determined the spore counts for each medium/carrier combination according to the provided spore enumeration protocol. The log spores per carrier are presented in Figure 1. The mean log spore count values across laboratories for each medium/carrier combination were above the target minimum of 5 logs of spores per carrier (Table 6). Conventional hypothesis testing showed that the mean log spores per carrier for SENB/PC was statistically significantly different from the mean for NA/PC ($P = 0.02$, SENB/PC higher by 0.61 logs). Mean log spores per carrier for SENB/PC and NA/SS were not statistically different ($P = 0.22$, SENB/PC higher by 0.35 logs). For an equivalence zone where the difference in means is no greater than ±1 (e.g., $\delta_a = 1$), both NA/PC and NA/SS were statistically equivalent to SENB/PC at a 0.05 level of significance. Although the data discredit the null hypothesis of no difference between the means of SENB/PC and NA/PC, the difference is judged to be of no practical importance when compared to the equivalence zone $-1.0 < \delta < 1.0$.

It should be noted that adjustment of the NA spore preparations used to inoculate PC and SS carriers to arrive at carrier counts statistically the same as those for SENB/PC was not emphasized by the Study Director; rather, the goal of achieving counts within the target range was prioritized. It would be possible to adjust the NA spore preparation by dilution or concentration to increase or decrease the carrier count; however, the goal of the study was to confirm the practicality of meeting the recommended target carrier load without additional manipulation of the NA spore preparation. Based on the Study Director's experience with carrier-based qualitative tests, the statistical difference in carrier counts noted for SENB/PC and NA/PC, 0.61 logs, is likely not of practical importance to the pass/fail outcome of AOAC Method **966.04**, i.e., the sensitivity of the method is not high enough to be affected by this difference in carrier counts. *Note*: The Study Director assumed that the outcome of the comparative studies (e.g., HCl resistance, efficacy, wash-off) for SENB/PC versus NA/PC was not impacted by the difference in carrier counts.

Table 6 presents the SD_R for each medium/carrier combination. The reproducibility is good relative to other standardized antimicrobial tests (17). In this respect, each medium/carrier combination consistently produced an acceptable number of spores across laboratories, thus providing support for the enumeration procedure and the target spore count as modifications to Method **966.04**.

HCl Resistance

The test for HCl resistance was conducted on each medium/carrier combination by each laboratory. AOAC Method **966.04** calls for a minimum of 2 min resistance of spores to 2.5 M HCl, i.e., growth of *B. subtilis* in the recovery medium after 2 min exposure to HCl is considered a pass and lack of growth is considered a fail. In this study, each test resulted in an outcome of pass; each laboratory recorded growth in at least the secondary recovery medium after 2 min exposure for each medium/carrier combination (Table 7). The pattern of positive tubes (i.e., positive at 2, 5, and 10 min) was variable across the tests. This is not

Table 6. Statistical summary of log_{10} spores per control carrier

Medium/carrier[a]	Mean log spores per carrier	SD_R[b]	p[c]	Mean difference (S–N) and 90% confidence limits
SENB/PC	5.87	0.401	—	—
NA/PC	5.26	0.181	0.02	0.61 (0.30 to 0.93)
NA/SS	5.52	0.433	0.22	0.35 (–0.19 to 0.89)

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); NA/SS (amended nutrient agar/stainless steel).

[b] SD_R = Reproducibility standard deviation.

[c] Comparing mean log_{10} spore counts per carrier for SENB/PC to NA/PC and NA/SS.

unexpected and falls within the range of what would be considered typical for this assay.

Neutralization Confirmation

Two laboratories conducted the neutralization confirmation procedure. Results are presented in Tables 8–10. The laboratories generated comparable results. For each test chemical/neutralizer combination, growth of *B. subtilis* occurred in both the primary and secondary treatments at inoculum dilutions providing low levels of spore challenge (5–100 spores/mL). Acceptable results were recorded for the inoculated controls and uninoculated controls (data not shown). The data support the selection and effectiveness of letheen broth + 0.1% sodium thiosulfate as a neutralizer for sodium hypochlorite, and FTM as a neutralizer medium for the peracetic acid/hydrogen peroxide and glutaraldehyde. This procedure is relevant to Method **966.04**, is simple to conduct, and provides valuable quantitative information on neutralizer effectiveness and the occurrence of bacteriostatic activity. This procedure will be a valuable addition to Method **966.04**.

Efficacy

Pass/fail results.—A comparison of results for the current and modified methods is shown in Tables 11 and 12. The results are presented as pass (zero positive carriers/30) or as fail (≥1 positive carrier/30). The number of positive carrier sets with growth of *B. subtilis* is also provided.

The percentages of overall agreement between pass/fail results for 24 total tests (2 test conditions for each of 3 sporicides tested in each of 4 laboratories) were 96% between SENB/PC and NA/PC and 88% between SENB/PC and NA/SS. Each NA method had 100% agreement with a passed SENB/PC outcome. The NA/PC and NA/SS methods had 93 and 80% agreement, respectively, with a failed SENB/PC outcome. Overall, the high percent agreement of the current to the proposed modified methods supports the

Table 7. Outcome of the HCl resistance test for current and modified AOAC Method 966.04

Lab	Medium/carrier combination[a]	Outcome[b] (pass/fail)	Exposure period and occurrence of tubes with growth (+) or no growth (–)			
			2 min	5 min	10 min	20 min
1	SENB/PC	Pass	–/+	–/–	–/–	–/–
	NA/PC	Pass	–/+	+/–	–/–	–/–
	NA/SS	Pass	+/+	–/–	–/–	–/–
2	SENB/PC	Pass	+/+	–/+	–/+	–/–
	NA/PC	Pass	–/+	–/–	–/–	–/–
	NA/SS	Pass	+/+	–/–	–/–	–/–
3	SENB/PC	Pass	+/+	–/–	–/–	–/–
	NA/PC	Pass	+/+	–/–	–/–	–/–
	NA/SS	Pass	+/+	+/+	–/–	–/–
4	SENB/PC	Pass	+/+	+/–	+/–	–/–
	NA/PC	Pass	+/+	+/+	–/–	–/–
	NA/SS	Pass	+/+	+/+	+/–	–/–

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); NA/SS (amended nutrient agar/stainless steel).

[b] Pass (spores must resist HCl for ≥2 min to be qualified as resistant test spores).

Table 8. Neutralization confirmation of bleach with letheen broth + 0.1% sodium thiosulfate

	Lab 1			Lab 2		
	Dilution					
	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
	Spores/mL					
Treatments/controls	91	13	2	101	12	1
Neutralizer-primary subculture[a]	+	+	+	+	+	–
Secondary subculture[b]	+	+	+	+	+	+
Neutralizer inoculated control	+	–	–	+	+	–
Subculture inoculated control	+	+	–	+	+	–

[a] Letheen broth + 0.1% sodium thiosulfate.

[b] Fluid thioglycollate medium (tubes contain a carrier).

Table 9. Neutralization confirmation of peracetic acid/hydrogen peroxide product with fluid thioglycollate medium

	Lab 1			Lab 2		
	Dilution					
	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
	Spores/mL					
Treatments/controls	91	13	2	113	8	1
Neutralizer-primary subculture[a]	+	+	+	+	–	+
Secondary subculture[b]	+	+	+	+	+	+
Neutralizer inoculated control	+	+	+	+	+	+
Subculture inoculated control	+	+	+	+	+	+

[a] Fluid thioglycollate medium.

[b] Fluid thioglycollate medium (tubes contain a carrier).

Table 10. Neutralization confirmation of glutaraldehyde with fluid thioglycollate medium

	Lab 1			Lab 2		
	Dilution					
	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
	Spores/mL					
Treatments/controls	89	12	1	108	8	1
Neutralizer-primary subculture[a]	+	+	–	+	+	–
Secondary subculture[b]	+	+	–	+	+	–
Neutralizer inoculated control	+	+	–	+	+	+
Subculture inoculated control	+	+	–	+	+	+

[a] Fluid thioglycollate medium.

[b] Fluid thioglycollate medium (tubes contain a carrier).

Table 11. Comparative efficacy results for low chemical treatments

Chemical treatment	Medium/carrier combination[a]	Outcome and number of positive carriers			
		Lab No. 1	Lab No. 2	Lab No. 3	Lab No. 4
Peracetic acid and hydrogen peroxide	SENB/PC	Fail (16+)	Fail (28+)	Fail (21+)	Fail (28+)
	NA/PC	Fail (29+)	Fail (17+)	Fail (28+)	Fail (30+)
	NA/SS	Fail (20+)	Fail (30+)	Fail (30+)	Fail (20+)
Glutaraldehyde	SENB/PC	Fail (15+)	Fail (9+)	Fail (5+)	Fail (23+)
	NA/PC	Fail (17+)	Fail (26+)	Fail (22+)	Fail (21+)
	NA/SS	Fail (3+)	Fail (27+)	Fail (1+)	Fail (29+)
Bleach	SENB/PC	Fail (13+)	Fail (20+)	Fail (16+)	Fail (29+)
	NA/PC	Fail (28+)	Fail (24+)	Fail (6+)	Fail (2+)
	NA/SS	Fail (3+)	Fail (22+)	Fail (11+)	Fail (5+)

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); and NA/SS (amended nutrient agar/stainless steel).

adoption of the modifications. *Note*: No additional positive tubes were recorded following the heat shock step. This step will not be included in the revised alternate method.

Low treatments.—Exposing carriers (30 carrier tests) to the low treatments resulted in a fail for 36 out of 36 tests (Table 11). The number of positive carriers ranged from 1 to 30 out of 30. Thus, the pass/fail outcome for NA/PC and NA/SS always agreed with SENB/PC, i.e., no discordant pairs were detected. Of the total positive carriers (684) across all low treatments and laboratories, 33, 36, and 31% were from SENB/PC, NA/PC, and NA/SS, respectively. These data strongly indicate that the modified methods, when used in place of the current method, will provide the same outcome for less effective formulations. There was no indication that the modifications created a less stringent test (i.e., NA/PC or NA/SS passed a test chemical when the SENB/PC failed the test chemical). In fact, based on the total percentage of total positives, the NA/PC was slightly higher, suggesting a more conservative method. Thus, this component of the study supports the use of NA/PC and NA/SS as viable replacements to SENB/PC.

High treatments.—The medium/carrier combinations exposed to the high treatments produced a pass result in 31 out of the 36 tests conducted. The number of positive carriers from the 5 failed tests was low: 1 to 3 positive carrier sets out of 30. The failed carrier sets occurred for the peracetic acid/hydrogen peroxide and bleach treatments.

With few exceptions, SENB/PC and the modified combinations, NA/PC and NA/SS, provided the same results

Table 12. Comparative efficacy results for high chemical treatments

Chemical treatment	Medium/carrier combination[a]	Outcome and number of positive carriers			
		Lab No. 1	Lab No. 2	Lab No. 3	Lab No. 4
Peracetic acid and hydrogen peroxide	SENB/PC	Fail (1+)	Pass (0+)	Pass (0+)	Pass (0+)
	NA/PC	Fail (1+)	Pass (0+)	Pass (0+)	Pass (0+)
	NA/SS	Pass (0+)	Pass (0+)	Pass (0+)	Pass (0+)
Glutaraldehyde	SENB/PC	Pass (0+)	Pass (0+)	Pass (0+)	Pass (0+)
	NA/PC	Pass (0+)	Pass (0+)	Pass (0+)	Pass (0+)
	NA/SS	Pass (0+)	Pass (0+)	Pass (0+)	Pass (0+)
Bleach	SENB/PC	Fail (2+)	Fail (2+)	Pass (0+)	Pass (0+)
	NA/PC	Fail (3+)	Pass (0+)	Pass (0+)	Pass (0+)
	NA/SS	Pass (0+)	Pass (0+)	Pass (0+)	Pass (0+)

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); and NA/SS (amended nutrient agar/stainless steel).

Table 13. Pass/fail outcome for SENB/PC compared to the NA/PC and NA/SS outcomes for the same treatment and laboratory; high chemical treatments

	Outcome for NA/PC[a]			Outcome for NA/SS[a]		
Outcome for SENB/PC[a]	Pass	Fail	Total	Pass	Fail	Total
Pass	9	0	9	9	0	9
Fail	1[b]	2	3	3[c]	0	3
Total	10	2	12	10	2	12

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); and NA/SS (amended nutrient agar/stainless steel).

[b] Discordance observed by Laboratory 2 when testing bleach.

[c] Discordance observed by Laboratory 1 when testing peracetic acid–hydrogen peroxide and by Laboratories 1 and 2 when testing bleach.

for the high treatments. The outcome for SENB/PC and NA/PC against the high treatments was the same for 11 of the 12 pairs (Tables 12 and 13). One discordant pair was recorded by Laboratory 2 (pass with NA/PC and a fail with SENB/PC when testing the high bleach). Three discordant pairs were recorded for SENB/PC and NA/SS against the high treatments, a pass for NA/SS was recorded 3 times when a fail was recorded for SENB/PC (Tables 12 and 13). The estimated differences [*P*-value (90% confidence interval)] between pass probabilities were –0.08 [*P* = 0.32, (–0.30, 0.12)] for SENB/PC minus NA/PC and –0.25 [*P* = 0.083, (–0.49, –0.02)] for SENB/PC minus NA/SS. Based on these findings for the 3 high chemical treatments combined, the SENB/PC was slightly more stringent than the 2 NA/carrier combinations.

Although the high treatments were designed to provide complete spore kill, the occurrence of the fail outcomes does not impact the quality of the collaborative study; rather it is likely due to inherent variability with the method and not associated with a specific method characteristic. In theory, a single viable spore can produce a tube with growth; the same result could also be generated by the occurrence of many survivors. The fail outcomes do not appear to be associated with significantly higher carrier counts, technical error, or improper preparation of the test chemicals. The modified methods do not appear to be more conservative than the current method; rather there is a slight tendency for the modifications to be less stringent, most notably for NA/SS. Overall, the high treatment data provide evidence strongly supporting the use NA/PC as a replacement for SENB/PC. The occurrence of the one discordance for the bleach treatment is recognized and is not considered to be of practical importance in this instance (2/30 positive for SENB/PC versus 0/30 positive for NA/PC). NA/SS has excellent potential as a replacement for SENB/PC as well, although the occurrence of the 3 discordant pairs indicates a less stringent test. NA/SS is the combination of 2 method modifications, NA and SS; thus, achieving equivalency to SENB/PC is more challenging. Similar to the use of NA as a replacement for SENB, the advantages of stainless steel over porcelain as a carrier material in Method **966.04** must also be considered in the final recommendation to revise the method.

LR results using the P/N formula.—The number of positive carriers for the high chemical treatments was usually zero; thus, the variability among LR is primarily due to the variability among control carrier counts. For this reason, the usefulness of presenting the P/N formula LRs to compare the equivalency of the medium/carrier types is limited and will not be presented for the high chemical treatments.

Figure 2 shows that there is no consistent relationship for the low chemical treatments between the number of spores per carrier and the number of positive carriers observed when testing a sporicide. It appears that greater number of fails observed with SENB/PC cannot be attributed to the slightly higher spores per carrier for SENB/PC. The range of the mean number of spores per carrier among the 3 methods is not wide enough to show an effect on the efficacy test outcome. These plots therefore support the conclusion that the observed differences in mean spores per carrier (Table 6) are not of practical importance.

The P/N formula was used to convert the number of positive carriers into an LR value. A statistical summary of the LR values for each medium/carrier combination is presented

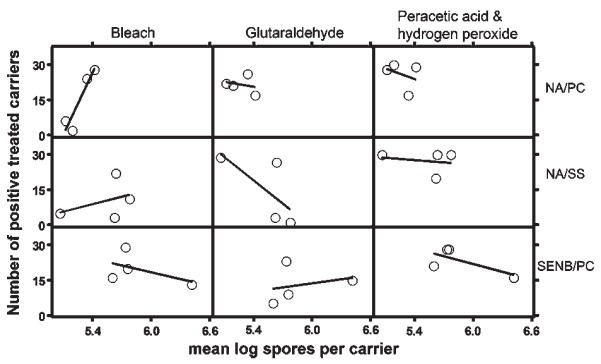

Figure 2. The 4 points in each panel are observations at 4 laboratories when testing the low chemical treatments. The same 4 laboratories tested each combination of medium/carrier and sporicide. The line in each panel is the least squares regression line.

Table 14. Comparison of log reduction (LR) estimates for the low treatments combined

Medium/carrier combination[a]	Mean LR	SD_R	p^b	Mean difference and 90% confidence limits
SENB/PC	5.90	0.535	—	—
NA/PC	5.20	0.416	0.01	0.69 (0.29 to 1.09)
NA/SS	5.64	0.757	0.16	0.26 (–0.05 to 0.57)

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); and NA/SS (amended nutrient agar/stainless steel).

[b] Comparing mean log reductions for SENB/PC to NA/PC and NA/SS.

in Table 14. The SENB/PC – NA/PC difference between LR values was 0.69, which was statistically significantly different from zero ($P = 0.01$). In this case, the LR estimate for NA/PC is lower, thus providing a more conservative test than SENB/PC. The SENB/PC – NA/SS difference between LR values was only 0.26, which was not statistically significantly different from zero ($P = 0.16$). For an equivalence zone where the difference in mean LRs is no greater than ±1 (e.g., $\delta_a = 1$), NA/SS was statistically equivalent to SENB/PC at a 0.05 level of significance. Although NA/PC was not formally statistically equivalent to SENB/PC, the 90% confidence interval extended outside the equivalence zone by only 0.09 (Table 14).

Quantitative Efficacy

In this study, the low chemical treatments were selected to provide a measurable population of survivors for comparison purposes. The LR values for each medium/carrier combination were compared. Carriers used in this experiment were from the same preparations of carriers used throughout the collaborative study; thus, the control carrier counts previously discussed in the carrier count section were used in the LR calculations.

Following exposure, the carriers were handled according to Method **966.04** except for the following: (*1*) Treated carriers were placed into 10 mL water and not into a specific neutralizer medium; and (*2*) carriers were processed quantitatively according to the carrier count procedure. One mL aliquots of the 1.0×10^{-1}–1.0×10^{-4} dilutions (serial dilution blanks with recovered spores) were plated. Wash-off of spores in the test chemical tube was not accounted for in this study.

Spores counts were less than detectable for several chemical treatment × medium/carrier combinations (Table 15), especially for bleach. Viable spores may have been recovered if the 1.0×10^0 dilution had also been plated. Due to the high frequency of zero spores recovered from the bleach treatment, only data from the glutaraldehyde and peracetic acid/hydrogen peroxide treatments were analyzed for medium/carrier equivalency.

The statistical analysis was conducted on paired combinations of SENB/PC versus NA/PC and SENB/PC versus NA/SS. There were 3 pairs of SENB/PC and NA/PC LRs and 4 pairs of SENB/PC and NA/SS. Mean LR values of 2.92, 2.51, and 2.47 were generated for glutaraldehyde for SENB/PC, NA/PC, and NA/SS, respectively. For the peracetic acid–hydrogen peroxide treatment, LR values of 3.06, 1.57, and 4.01 were observed for SENB/PC, NA/PC, and NA/SS, respectively. Across both chemicals, the mean LR differences for SENB/PC – NA/PC and SENB/PC – NA/SS were 0.60 and –0.25, respectively. The differences in the mean LR values were not statistically significant different from zero (Table 16). For an equivalence zone where the difference in means is no greater than ±1 (e.g., $\delta_a = 1$), both NA/PC and NA/SS were not statistically equivalent to SENB/PC at a 0.05 level of significance. However, there is little power to

Table 15. Mean log spores per carrier recovered for each medium/carrier type combination following exposure to the low chemical treatments

	Bleach		Glutaraldehyde		Peracetic acid–hydrogen peroxide	
Medium/carrier combination[a]	Lab 2	Lab 3	Lab 2	Lab 3	Lab 2	Lab 3
SENB/PC	LTD[b]	3.36	3.07	2.44	2.19	3.03
NA/PC	LTD	LTD	3.60	1.82	LTD	3.55
NA/SS	LTD	2.10	3.82	2.64	0.09	3.29

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); and NA/SS (amended nutrient agar/stainless steel).

[b] LTD = Less than detectable. For each of the 5 treated carriers, there were zero CFUs at every dilution.

Table 16. Statistical comparison of quantitative LR values for the 3 medium/carrier combinations; the LR were paired by laboratory and treatment (low treatments of glutaraldehyde and peracetic acid–hydrogen peroxide)

Medium/carrier combination[a]	Mean LR across treatments and labs	SD_R	p^b	Mean difference and 90% confidence limits
SENB/PC	2.99	0.553	—	—
NA/PC	2.04	1.105	0.25	0.60 (–0.48 to 1.68)
NA/SS	3.24	1.666	0.71	–0.25 (–1.68 to 1.18)

[a] SENB/PC (soil extract nutrient broth/porcelain); NA/PC (amended nutrient agar/porcelain); and NA/SS (amended nutrient agar/stainless steel).

[b] Comparison of mean LR values between SENB/PC and NA/PC, SENB/PC and NA/SS.

show equivalence for these data because each 90% confidence interval was very wide due to the small number of differences being averaged. Based on the analysis of LR values generated in this study, the current and modified methods are comparable, thus providing additional information to support NA and SS as official modifications to Method **966.04**.

Wash-Off in Saline

Spore wash-off was investigated as an indicator of differences in adhesion of spores to the carrier surface after exposure to liquid. Three carriers of each medium/carrier combination were exposed to 2 saline treatments (0.85% saline and 0.85% saline + polysorbate 80). The saline + polysorbate 80 (final concentration 0.01%) was used to increase the surfactancy of the saline, thus simulating an actual product. Unlike the exposure of 5 carriers per 10 mL solution in efficacy testing, carriers were analyzed separately in this study. Carriers used in this study were selected from the same pool of carriers used to conduct other studies in this collaborative.

The geometric mean percentage wash-off for the saline treatment (4.7%) was larger than for saline + polysorbate treatment (4.0%), but the ratio was not statistically significantly different from 1.0 (P = 0.71) The 90% confidence interval for the ratio of geometric means, S to S + P, was (0.5, 2.8). Because there was no practical difference between the treatments, subsequent analyses pooled the data over S and S + P.

In this experiment, the amount of wash-off was low for the 3 medium/carrier combinations. Table 17 shows the statistical summary of wash-off percentages for each medium/carrier. The geometric mean percentage washed-off (averaged over S and S + P and laboratories) was higher for SENB/PC (30%) than for NA/PC (8%) and NA/SS (0.3%). The mean wash-off for NA/SS was statistically significantly different from SENB/PC (P = 0.007). The small amount of wash-off observed for the NA/SS method is considered an advantageous characteristic for a carrier-based test. Because the mean wash-off percentages for all 3 methods were so low, a formal equivalence test was not necessary.

The total spores used as the denominator in the percentage washed-off calculation amounted to a 2-step calculation of the number of spores per control carrier. It is of interest to compare the counts of total spores in this special task to the carrier counts observed for the main study. Table 18 shows the statistical summary for the log total spores. The geometric means of the total number of spores per carrier were 4.6×10^5 for SENB/PC, 1.3×10^5 for NA/PC, and 2.9×10^5 for NA/SS. For an equivalence zone where the difference in means is no greater than ±1 (e.g., $\delta_a = 1$), both NA/PC and NA/SS were statistically equivalent to SENB/PC at a 0.05 level of significance. The limited data in this special task agree with the main results in that the 3 medium/carrier methods attained the target of 5 logs for the number of spores per control carrier.

Table 17. Comparison of wash-off percentages for the medium/carrier combinations; all statistical calculations were conducted using the log_{10}-transformed wash-off percentages

Medium/carrier combination	Geometric mean wash-off percentage, averaged across treatments and labs, %	SD_R	p^a	Ratio of geometric means and 90% confidence limits
SENB/PC	30	20.14	—	—
NA/PC	8	16.42	0.28	4 (0 to 38)
NA/SS	0.3	0.56	0.007	89 (18 to 450)

[a] Two-tailed p-value for a paired t-test based on log_{10} percentages; the null hypothesis is that the ratio of geometric mean wash-off percentages, SENB/PC to NA, equals 1.

Table 18. Comparison of log total spores for the medium/carrier combinations

Medium/carrier combination	Mean of log total spores per carrier	SD_R	p^a	Difference between means of log total spores and 90% confidence limits
SENB/PC	5.66	0.11	—	—
NA/PC	5.10	0.28	0.039	0.56 (0.18 to 0.94)
NA/SS	5.47	0.30	0.38	0.19 (–0.25 to 0.63)

[a] Two-tailed *p*-value for a paired *t*-test based on $\log_{10}$ percentages; the null hypothesis is that the ratio of geometric mean wash-off percentages, SENB/PC to NA, equals 1.

Conclusions

The collaborative study was undertaken to compare the current and modified methods for **966.04** and determine if the methods are statistically equivalent. To generate data, a series of comparative experiments were conducted on the set of core modifications, i.e., use of NA for spore production and SS carriers. Each experiment provided specific data relevant to Method **966.04,** and in combination, was used to determine method equivalency. Also, new procedures were introduced and evaluated, including a spore enumeration and neutralization confirmation procedure. Both were necessary to support the overall study and were under consideration as additions to Method **966.04**. On the basis of the results of this study, it is recommended that NA, the spore enumeration procedure, the target carrier count, and the neutralization confirmation procedure be adopted as Official First Action procedure modifications to Method **966.04**.

The results of the HCl resistance, efficacy, and wash-off studies demonstrate that NA in conjunction with the PC is comparable to the current method, SENB/PC. The NA method is simple, inexpensive, reproducible, and provides an ample supply of high quality spores. Based on the titer of spore suspensions (data not shown) and the associated control carrier counts, the NA method is a highly effective sporulation medium. Although the mean carrier counts for NA/PC were determined to be significantly lower than SENB/PC (difference of 0.61 logs), the counts were adequate (above 5 logs) and the difference was determined not to be of practical importance. In addition, the SD_R values were small (range of 0.43 for NA/SS to 0.18 for NA/PC) for the control carriers compared to other published antimicrobial tests. Method **966.04** does not specify a standard number of spores per carrier, maximum or minimum, or a procedure to enumerate spores from carriers; both aspects are considered major deficiencies. In this study, a spore enumeration procedure was introduced and successfully utilized; the procedure will augment Method **966.04** and allow the user to monitor carrier counts. Furthermore, the establishment of a mean spore titer per carrier was proposed (minimum of 5 logs and a maximum of approximately 6 logs); the target spore counts were shown to be obtainable. Currently, Method **966.04** does not provide a procedure to confirm neutralizer effectiveness. A neutralization confirmation procedure that simulates Method **966.04** was proposed and successfully conducted by 2 laboratories in this study. SS, in conjunction with NA, performed similarly to PC in the HCl resistance and efficacy tests (although a trend for less stringency was noted for SS); however, based on the wash-off studies, SS retained a significantly higher number of spores than PC (wash-off of 0.3% for SS vs 30% for PC). Although the data support the use of stainless steel for *B. subtilis*, due to the current use of porcelain carriers for testing *C. sporogenes*, it is advisable to retain the use of porcelain carriers until stainless steel can be evaluated as a replacement carrier material for *Clostridium*. The evaluation of stainless steel for *Clostridium* has been initiated by the Study Director.

A First Action Modified Method **966.04**, which includes the recommendations discussed, is presented in this report. The modified method also contains a series of editorial revisions proposed by the Study Director. The editorial changes are classified as minor changes. A minor change in a procedural parameter is one that, in the judgment of the General Referee and the Methods Committee, does not affect the method's performance and does not require new in-house data, although literature or historical data may exist. Editorial revisions include the following: (*1*) guidance on the safe handling of pathogenic microorganisms; (*2*) use of reagent grade water and commercial dehydrated media; (*3*) procedures for the maintenance of *B. subtilis* stock cultures and purity check; (*4*) intervals for time and temperature; (*5*) recording results for both primary and secondary subculture tubes as a carrier set; and (*6*) use of a certified timer for managing timed activities. Based on its lack of usefulness, a heat shock step for negative tubes is not incorporated into the modified method.

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Anthony Garza, EPA, Office of Pesticide Programs Microbiology Laboratory, Fort Meade, MD

Allison Rodriguez and Theresa To, FDA, Winchester Engineering and Analytical Center, Winchester, MA

Marie Buen, Elizabeth Gonzales, Kevin Kallander, and Patricia Stahnke, FDA, Denver District Laboratory, Denver, CO

Harriet Chan Myers, Advanced Sterilization Microbiology Laboratory, Irvine, CA

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AOAC Official Method 966.04
Sporicidal Activity of Disinfectants
Revised First Action 2006
Method II

(Applicable to testing sporicidal activity of liquid disinfectants using modified Method **966.04** against *Bacillus subtilis* on a hard surface (porcelain carrier). Performance criteria for product efficacy are not impacted. This method has been validated for products containing sodium hypochlorite, peracetic acid/hydrogen peroxide, and glutaraldehyde. *See* results of the collaborative study supporting the modifications to Method **966.04**.)

Caution: (*1*) Perform all manipulations of the test organism in accordance with biosafety practices stipulated in the institutional biosafety regulations. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms refer to the CDC/NIH *Biosafety in Microbiological and Biomedical Laboratories* manual. (*2*) Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Use personal protective clothing or devices during the handling of these items for purpose of activation, dilution, or efficacy testing. Use a chemical fume hood or other containment equipment when appropriate during performing tasks with concentrated products. Consult the *Material Safety Data Sheet* for the specific product/active ingredient to determine best course of action. (*3*) References to water mean reagent grade water, except where otherwise specified. (*4*) Commercial dehydrated media made to conform to the specified recipes may be substituted. (*5*) These microbiological methods are very technique-sensitive and technique-oriented; thus, exact adherence to the method, good laboratory practices, and quality control (QC) are required for proficiency and validity of the results. (*6*) Detergents used in washing glassware may leave residues which are bacteriostatic. Test for inhibitory residues on glassware periodically. For procedure, refer to *Standard Methods for the Examination of Water and Wastewater*, Section 9020, Quality Assurance/Quality Control.

A. Media and Reagents

(**a**) *Culture Media.*—(*1*) *Nutrient broth*.—For use in preparing nutrient agar. Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (anatone, peptic hydrolysate of pork tissues, manufactured by American Laboratories, Inc., 4410 S. 102nd St, Omaha, NE 68127) to approximately 1 L water. Boil mixture for 20 min with constant stirring. Readjust volume to 1 L with water and cool to around 50°C. Adjust pH to 6.8 ± 0.2 with 1 N HCl or 1 N NaOH, if necessary. Filter through paper (e.g., Whatman No. 4). Dispense 10 mL portions into 20 × 150 mm culture tubes or 20 mL portions into 25 × 150 mm culture tubes. Dehydrated nutrient broth may be substituted; prepare according to the manufacturer's instructions. (*2*) *Nutrient agar*.—For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized nutrient broth. Boil mixture until agar is dissolved. Adjust pH to 7.2 ± 0.2 if necessary. Dispense 5 mL portions into 16 × 100 mm screw-cap tubes. Larger tubes may be used as well. Autoclave for 20 min at 121°C. Remove from autoclave and slant tubes to form agar slopes. (*3*) *Nutrient agar with 5 μg/mL $MnSO_4 \cdot H_2O$ (amended nutrient agar)*.—For spore production. Suspend 11.5 g nutrient agar in 495 mL water, and add 5 mL 500 ppm $MnSO_4 \cdot H_2O$. Dissolve by boiling. Adjust pH to 6.8 ± 0.2 if necessary. Autoclave for 15 min at 121°C. Pour agar into plates. (*4*) *Trypticase soy agar (TSA)*.—Suspend 40 g dehydrated TSA in 1 L water and heat

gently while stirring. Boil 1 min or until completely dissolved. Adjust pH to 7.3 ± 0.2. Autoclave 15 min at 121°C. Pour agar into plates. (*5*) *Fluid thioglycollate medium (FTM)*.—Suspend 29.5 g dehydrated FTM in 1 L water. Heat to boiling to dissolve completely. Adjust pH to 7.1 ± 0.2 if necessary. Dispense 10 mL portions into 20 × 150 mm culture tubes and autoclave for 15 min at 121°C. Store at room temperature. Protect from light. *Note*: If after autoclaving the aerated portion of media consumes more than one-third of tube, media must be reboiled by placing tubes in beaker of boiling water. Media can only be reboiled once. (*6*) *FTM with 1 M NaOH (modified FTM).*—For subculturing spores exposed to 2.5 M HCl. Suspend 29.5 g FTM in 1 L water. Heat boiling to dissolve completely. Cool and adjust pH to 7.1 ± 0.2 if necessary. Add 20 mL 1 M NaOH, and mix well. Check final pH and record (pH between 8 and 9 is typical). Dispense 10 mL into 20 × 150 mm culture tubes and autoclave for 15 min at 121°C. Store at room temperature. Protect from light. *Note*: If after autoclaving the aerated portion of media consumes more than one-third of tube, media must be reboiled by placing tubes in beaker of boiling water. Media can only be reboiled once. *Note*: Media can be stored for up to 2 months.

(**b**) *Manganese sulfate monohydrate*.—500 ppm. Add 0.25 g $MnSO_4 \cdot H_2O$ to 500 mL water. Filter-sterilize for use.

(**c**) *Dilute hydrochloric acid*.—2.5 M. Use to determine resistance of dried spores. Standardize and adjust to 2.5 M as in **936.15** (*see* A.1.06).

(**d**) *Sterile water.*—Use reagent grade water. Reagent grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent grade water when used in the proper arrangement. *See Standard Methods for the Examination of Water and Wastewater* for details on reagent grade water.

(**e**) *Triton X-100*.

(**f**) *Ethanol (40%)*.

(**g**) *Test organism.—Bacillus subtilis* (ATCC No. 19659) obtained directly from a reputable supplier (e.g., ATCC).

B. Apparatus

(**a**) *Carriers.*—Penicylinders, porcelain, 8 ± 1 mm od, 6 ± 1 mm id, 10 ± 1 mm length (available from CeramTec Ceramic, PO Box 89, Laurens, SC 29360-0089, www.ceramtec.com, Cat. No. LE15819).

(**b**) *Glassware*.—For disinfectant, 25 × 150 mm or 25 × 100 mm culture tubes (Bellco Glass Inc., Vineland, NJ; reusable or disposable 20 × 150 mm (for cultures/subcultures); 16 × 100 mm screw-cap tubes for stock cultures. Cap with closures before sterilizing. Sterilize all glassware 2 h in hot air oven at 180°C or steam-sterilize for a minimum of 20 min at 121°C with drying cycle.

(**c**) *Sterile centrifuge tubes*.—Polypropylene, 15 mL conical tubes with conical bottoms (Fisher Scientific, Pittsburgh, PA; Corning, Inc., New York, NY), or equivalent.

(**d**) *Water bath/chiller unit.*—Constant temperature for test chemical, capable of maintaining 20 ± 1°C or specified temperature for conducting the test.

(**e**) *Petri dishes*.—Plastic (sterile).

(**f**) *Filter paper*.—Whatman No. 2; placed in Petri dishes for storing carriers.

(**g**) *Test tube racks*.—Any convenient style.

(**h**) *Inoculating loop*.—Any convenient inoculation/transfer loop for culture transfer.

(**i**) *Wire hook*.—For carrier transfer. Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gauge with other end in suitable holder.

(**j**) *Centrifuge.*—Nonrefrigerated (e.g., Eppendorf 5804 R).

(**k**) *Sonicator.*—Ultrasonic cleaner (e.g., Branson, Danbury, CT; Model 1510).

(**l**) *Orbital shaker.*—Speed range from 25 to 500 rpm (e.g., VWR, Westchester, PA; DS-500).

(**m**) *Vacuum desiccator*.—For carrier storage. With adequate gauge for measuring 27 in. (69 cm) of Hg and fresh desiccant.

(**n**) *Certified biosafety cabinet (BSC; Class I or II)*.— To maintain aseptic work environment.

(**o**) *Certified timer*.—For managing timed activities; any certified timer that can display time in seconds.

C. Operating Technique

(**a**) *Culture initiation*.—Initiate *B. subtilis* culture (e.g., use nutrient broth to rehydrate a lyophilized culture, and incubate the broth culture for 24 ± 2 h at 36 ± 1°C prior to streak inoculation). Streak inoculate a set (e.g., 6) nutrient agar slopes and incubate 24 ± 2 h at 36 ± 1°C. Concurrently, perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or other identification systems). After incubation, store at 2–5°C. Maintain stock culture on nutrient agar slants by monthly (30 ± 2 days) transfers.

(**b**) *Production of B. subtilis spore suspension*.—Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of nutrient broth and incubate tubes on an orbital shaker for 24 ± 2 h at ca 150 rpm at 36 ± 1°C. Use this culture to inoculate amended nutrient agar plates. Inoculate each plate with 500 µL broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12–14 days at 36 ± 1°C. After incubation, harvest the spores by adding 10 mL cold sterile water to each plate. Using a spreader (e.g., bent glass rod), remove growth from plates, and pipet suspensions into 15 mL sterile conical tubes (10 plates = 14 tubes, about 10 mL each). Centrifuge tubes at 5000 rpm for ca 10 min at room temperature. Remove and discard supernatant. Resuspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5000 rpm for ca 10 min. Remove and discard supernatant. Repeat twice. Resuspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2–5°C. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the

spores. Examine a minimum of 5 fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores vs vegetative cells should be at least 95%. Spore suspension from multiple plates can be combined and re-aliquoted into tubes for uniformity. Before inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0×10^{-6} through 1.0×10^{-8}) using pour or spread plating on TSA plates. For pour plating, add molten TSA tempered to 45–55°C to each plate, swirl, and allow agar to solidify. Incubate plates for 24 ± 2 h at 36 ± 1°C and determine titer. *Note*: When harvested and processed, 10 plates of amended nutrient agar should provide 80–100 mL of concentrated spore suspension (ca 10^9 colony-forming units (CFU)/mL). Diluting the suspension before carrier inoculation will be necessary; a titer of 1.0×10^8–5.0×10^8 CFU/mL should be adequate to achieve the target carrier count.

(**c**) *Preparation of porcelain carriers*.—Before use, examine porcelain carriers individually and discard those with scratches, nicks, spurs, or discolorations. Rinse unused carriers gently in water 3 times to remove loose material, and drain. Place rinsed carriers into Petri dishes matted with 2 layers of filter paper in groups of 15 carriers per Petri dish. Sterilize 20 min at 121°C. Cool and store at room temperature. *Note*: Handle porcelain carriers with care when placing in Petri dishes. Minimize carrier movement and avoid excessive contact between carriers that might result in chips and cracks. Wash carriers with Triton X-100, and rinse with water 4 times for reuse.

(**d**) *Inoculation of porcelain carriers*.—Dilute the concentrated spore suspension as necessary with sterile water to achieve carrier counts between 1.0×10^5 and ca 1.0×10^6 spores/carrier. Dispense 10 mL diluted spore suspension into an appropriate number of 25 × 150 mm tubes. Add 10 sterile carriers to each tube containing 10 mL spore suspension, slightly agitate, and let stand 10–15 min. Remove each carrier with sterile hook and place upright in sterile Petri dish lined with 2 sheets of filter paper, no more than 30 carriers per Petri dish. Air-dry in BSC, **B**(**n**), for ca 30 ± 2 min. Place Petri dishes containing inoculated carriers in vacuum desiccator containing $CaCl_2$ and draw vacuum of 69 cm (27 in.) Hg. Dry carriers under vacuum for 24 ± 2 h before use in HCl resistance, efficacy testing, or carrier counts. Maintain under vacuum for up to 3 months. Carriers may be used after 3 months if they meet the acceptable HCl resistance and carrier count criteria. Inoculated carriers should not be used after 1 year of storage. Sterilize and reuse if necessary [*see* **C**(**c**)].

(**e**) *Spore enumeration (carrier counts)*.—Before use, determine the carrier counts for each preparation of carriers. Assay 3–5 randomly selected carriers per preparation. Place each inoculated carrier into a 50 mL plastic, polypropylene conical centrifuge tube containing 10 mL sterile water. Sonicate carriers for 5 min ± 30 s. *Note*: For sonication, place tubes into an appropriately sized beaker with tap water to the level of sterile water in the tubes. Place beaker in sonicator so that water level in the beaker is even with water level fill line on sonicator tank. Fill tank with tap water to water level fill line. Suspend beaker in sonicator tank so that it does not touch bottom of tank and all 3 water levels (inside test tubes, inside beaker, and sonicator tank) are the same. After sonification, mix tubes in a Vortex mixer for 2 min ± 5 s. Dilute spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile water. Dilute spore suspensions to 1.0×10^{-5} and plate dilutions 1.0×10^{-2} through 1.0×10^{-5}. Plate each dilution in duplicate using pour or surface spread plating with TSA. For pour plating, add molten TSA tempered to 45–55°C to each plate. Swirl pour plates to distribute spores evenly, and allow agar to solidify. Invert plates and incubate for 24–48 h at 36 ± 1°C. Count colonies (by hand or with colony counter). Use dilutions yielding between 30 and 300 CFU per plate (target counts) for enumeration; however, record all counts less than 30. Report plates with colony counts over 300 as too numerous to count (TNTC). Average spore counts per carrier should be between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Do not use carriers with counts outside this range.

(**f**) *HCl resistance*.—Equilibrate water bath to 20 ± 1°C. Pipet 10 mL 2.5 M HCl into two 25 × 100 mm tubes, place in water bath, and allow to equilibrate. Start timer and rapidly transfer 4 inoculated penicylinders into an acid tube (2.5 M HCl) with flamed hooks or forceps. Do not allow carriers or transfer device to contact inside of wall of acid tube. Transfer individual carriers after 2, 5, 10, and 20 min of HCl exposure to a separate tube of modified FTM. Rotate each tube vigorously by hand for ca 20 s and then transfer carrier to a second tube of modified FTM. For viability control, place 1 unexposed inoculated carrier in a separate tube of modified FTM. For media sterility, use 1 tube of modified FTM. Incubate all test and control tubes for 21 days at 36 ± 1°C. Record results as growth (+) or no growth (–) at each time period. Spores should resist HCl for ≥2 min to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described.

(**g**) *Efficacy test.*–Aseptically prepare disinfectant test samples as directed. Prepare all dilutions with sterile calibrated volumetric glassware. For diluted products, use 1.0 mL or 1.0 g test disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. For a 30 carrier test, place 10 mL product at dilution recommended for use or under investigation into each of six 25 × 150 mm or 25 × 100 mm test tubes, or use appropriate number of tubes assuming 5 test carriers per tube of test chemical. Place tubes in 20 ± 1°C water bath and let equilibrate to temperature. Using a sterile hook (or forceps), transfer inoculated carriers sequentially at 2 min intervals in groups of 5 from Petri dish to test tubes containing sporicidal agent. Use a certified timer to monitor time. Flame hook and let cool after each transfer. When lowering carriers into test tube, neither carriers nor wire hook may touch sides of tubes. If interior sides are touched, note tube number. Do not count carrier set if any carrier from that group of 5 yields a positive result; test another set of 5 carriers. Carriers must be deposited into test tubes within ±5 s of the prescribed drop time. Return tubes to water bath immediately after adding carriers. After

contact period has been achieved, transfer carriers in same sequential timed fashion into primary subculture tubes containing appropriate neutralizer (10 mL in 20 × 150 mm test tubes). Remove the carriers one at a time from the test tube with sterile hook, tap against interior side of tube to remove excess sporicidal agent, and transfer into neutralizer tube (primary tube). All 5 carriers must be transferred during each 2 min interval. Flame hook between each carrier transfer. Move remaining carriers into their corresponding neutralizer tubes at appropriate time. Carriers may touch interior sides of neutralizer tube during transfer, but minimize contact. After each carrier is deposited, recap neutralizer tube and gently shake to facilitate adequate mixing and efficient neutralization. Within 1 h from when last carrier was deposited into primaries, transfer carriers using sterile wire hook to second subculture tube (secondary tube) containing 10 mL appropriate recovery medium, 1 carrier per tube. Move carriers in order, but movements do not have to be timed. Gently shake entire rack of secondary tubes after all carriers have been transferred. Incubate primary (neutralizer) and secondary subculture tubes for 21 days at 36 ± 1°C. Report results as growth (+) or no growth (–). A positive result is one in which medium appears turbid. A negative result is one in which medium appears clear. Shake each tube prior to recording results to determine presence or absence of growth/turbidity. Primary and secondary subculture tubes for each carrier represent a carrier set. A positive result in either primary or secondary subculture tube is considered a positive result for the carrier set.

Media sterility controls and system controls (check for aseptic technique during carrier transfer process) are recommended. For media controls, incubate 1–3 unopened subculture medium tubes with the test sample tubes for 21 days at 36 ± 1°C. For system controls, use sterile forceps or needle hooks to transfer 3 sterile carriers into a tube of test chemical. Transfer system control carriers to neutralizer medium as follows: at start of test (prior to first tube), transfer 1 sterile carrier to tube of neutralizer medium. After one-half of test carriers have been transferred to neutralizer tubes, transfer a second sterile carrier to tube of neutralizer medium. After all test carriers (last tube) have been transferred to neutralizer tubes, transfer third sterile carrier to tube of neutralizer medium. Transfer system control carriers to secondary subculture medium as follows: immediately before initiating transfer of test carriers into secondary subculture medium tubes, transfer first system control sterile carrier from neutralizer medium to tube of subculture medium. After one-half of test carriers have been transferred to secondary subculture medium tubes, transfer second system control sterile carrier to tube of subculture medium. After all test carriers have been transferred to secondary subculture medium tubes, transfer third system control sterile carrier to tube of subculture medium. For each test, include a positive carrier control by placing 1 inoculated carrier into tube of secondary subculture medium. Incubate controls and test sample tubes together for 21 days at 36 ± 1°C.

Perform identification confirmation on a minimum of three positive carrier sets per test, if available, using Gram stain and/or plating on TSA. Additional confirmation may be performed using VITEK, API analysis, or comparable method. If fewer than 3 positive carrier sets, confirm growth from each positive carrier set. If both tubes are positive in a carrier set, select only 1 tube for confirmatory testing. For test with 20 or more positive carrier sets, confirm at least 20% by Gram stain. If Gram stains are performed from growth taken directly from positive tubes, the staining should be performed within 5–7 days of conducting the efficacy test.

(**h**) *Neutralization confirmation procedure.*—A neutralization confirmation test must be performed in advance or in conjunction with efficacy testing. This assay is designed to simulate the conditions (i.e., neutralizer, subculture medium, contact time, diluent, concentration of test substance) of the efficacy test and to demonstrate the recovery of a low level of spores (e.g., 5–100). Diluted inoculum (e.g., spores of *B. subtilis*) is added directly to the various sets of subculture media tubes (*see* Table 4). This assay provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer–disinfectant interactions.

Produce a spore preparation according to the procedure for amended nutrient agar. Harvest growth from plates (e.g., 5 plates) per the method, except re-suspend pellet after final centrifugation step in approximately 100 mL aqueous (40%) ethanol. Determine spore count by serial dilution and plating on TSA. Desirable target of the initial working suspension is 1.0×10^8 to 1.0×10^9 CFU/mL. The suspension may require adjustment to reach target titer. Prepare serial 10-fold dilutions of the inoculum in sterile water out to 10^{-8}. Use 10^{-6}, 10^{-7}, and 10^{-8} dilutions to inoculate the neutralizer and subculture media tubes. The target number of spores to be delivered per tube in this assay is 5–100 per tube. Determine spore titer by plating (spread plate or pour plate) each of 3 dilutions in duplicate on TSA. Incubate plates inverted for 24–48 h at 36 ± 1°C. Count colonies (by hand or with colony counter). Report plates with colony counts over 300 as TNTC. *Note*: A standardized spore preparation adjusted to deliver 5–100 spores/mL may be substituted for the 3 dilutions of spore inoculum. In addition, spores sheared from inoculated carriers may be used as a working suspension.

Use 5 sterile porcelain carriers (only 3 to be used in the assay). Within 5 s, place a set of 5 carriers into a test tube (25 × 150 mm or 25 × 100 mm) containing test chemical; transfer carriers according to (**g**). Allow carriers to remain in test chemical per the specified contact time and temperature. After the contact time is complete, aseptically transfer 3 of the 5 carriers individually into tubes containing the neutralizer per (**g**). This set of tubes is the neutralizer/primary subculture treatment. After transfering the last carrier into neutralizer tube, transfer each carrier, in sequence, into a tube containing secondary subculture medium. This portion of assay is not timed, but should be made as soon as possible. This set is the secondary subculture treatment. After carrier transfer, inoculate each tube (neutralizer/primary and secondary

subculture treatment tubes) with 1 mL of each of 3 inoculum dilutions (10^{-6}, 10^{-7}, and 10^{-8}). For controls, use 3 fresh, unexposed tubes of neutralizer and 3 tubes of the secondary subculture medium; also inoculate each control tube with 1 mL of each of 3 inoculum dilutions. Include 1 uninoculated tube of neutralizer and secondary subculture media to serve as sterility controls. *See* Table 4 for tube inoculation scheme. Incubate all tubes 5–7 days at 36 ± 1°C. Record results as growth (+) or no growth (–). *Note*: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

Confirm a minimum of one positive tube per treatment and control (if available) using Gram staining and colony morphology on TSA. For each treatment and control group, conduct confirmation testing on growth from tube with fewest spores delivered. *B. subtilis* is a Gram-positive rod, and colonies on TSA are opaque, rough, dull, round, with irregular margins, and low convex. Colonial variation may be observed and is typical for this strain. Growth in the inoculated controls verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *Note*: There may be cases when the neutralizer is significantly different from the secondary subculture media; in these cases, growth may not be comparable. The uninoculated control tubes are used to determine sterility, and must show no growth for the test to be valid.

The occurrence of growth in the neutralizer/primary subculture and secondary subculture treatment tubes is used to assess the effectiveness of the neutralizer. No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For a neutralizer to be deemed effective, growth must occur in the Secondary Subculture treatment tubes which received lower levels of inoculum (e.g., 5–100 CFU/mL). Growth in the secondary subculture inoculated control verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance. Growth in the neutralizer-primary inoculated control should be comparable to the secondary subculture inoculated control if the neutralizer is the same as the secondary subculture media. There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the secondary subculture inoculated control. The neutralizer-primary and secondary subculture uninoculated control tubes are used to determine sterility, and must show no growth for the test to be valid.

Note: For product registration, the EPA requires the following to demonstrate sporicidal/sterilant-level efficacy: Using AOAC Method **966.04**, 60 carriers representing each of 2 types of surfaces (porcelain penicylinders and silk suture loops) must be tested separately against spores of both *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 3584) on 3 test samples representing 3 different batches of product, one of which must be at least 60 days old (2 carrier types × 2 test microorganisms × 60 carriers/type = 240 carriers per batch sample; 3 product batches × 240 carriers/batch = total of 720 carriers). The product must kill all of the test spores on all of the 720 carriers without any failures.

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