MICROBIOLOGICAL METHODS

Determining the Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard Nonporous Surface Using the Quantitative Three Step Method: Collaborative Study

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A collaborative study was conducted to validate the quantitative Three Step Method (TSM), a method designed to measure the performance of liquid sporicides on a hard nonporous surface. Ten laboratories agreed to participate in the collaborative study; data from 8 of 10 participating laboratories were used in the final statistical analysis. The TSM uses 5 5 1 mm glass coupons (carriers) upon which spores have been inoculated and which are introduced into liquid sporicidal agent contained in a microcentrifuge tube. Following exposure to a test chemical and a neutralization agent, spores are removed from carriers in 3 fractions: passive removal (Fraction A), sonication (Fraction B), and gentle agitation (Fraction C). Liquid from each fraction is serially diluted and plated on a recovery medium for spore enumeration. Control counts are compared to the treated counts, and the level of efficacy is determined by calculating the $\log_{10}$ reduction (LR) of spores. The main statistical goals were to evaluate the repeatability and reproducibility of the LR values, to estimate the components of variance for LR, and to assess method responsiveness. AOAC Method 966.04–Method II was used as a reference method. The scope of the validation was limited to testing liquid formulations against spores of *Bacillus subtilis*, a surrogate for virulent strains of *B. anthracis*, on a hard nonporous surface (glass). The test chemicals used in the study were sodium hypochlorite, a combination of peracetic acid and hydrogen peroxide, and glutaraldehyde. Each test chemical was evaluated at 3 levels of presumed efficacy: high, medium, and low. Three replications were required. The TSM was validated as it successfully met the statistical parameters for quantitative test methods. Satisfactory validation parameters, such as the repeatability standard deviation (S_r) and reproducibility standard deviation (S_R), were obtained for control carrier counts and LR values. Both the TSM and the reference method were responsive to the efficacy levels of the test chemicals. For the 72 total TSM tests conducted, the mean (± standard error of the mean) log density of spores per control carrier was 6.86 (± 0.08); the S_r and S_R were low at 0.15 and 0.27, respectively. Across the range of test chemicals, the S_r and S_R estimates associated with LR were also acceptably low. The S_r ranged from 0.17 to 0.72 and the S_R ranged from 0.34 to 1.43. Overall, the S_r and S_R estimates associated with the efficacy data were within the ranges published for other quantitative methods and meet the performance characteristics necessary for validation.

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Developing proven standard methods for evaluating the effectiveness of antimicrobial products, such as those used to decontaminate facilities intentionally contaminated in 2001 with spores of *Bacillus anthracis* (anthrax), is critical for protecting public health. Homeland Security Presidential Directive 10 (1) directs the United States Environmental Protection Agency (EPA) to take the federal lead for developing specific standards, protocols, and capabilities to address the risks of contamination following a biological weapons attack, and to develop strategies,

guidelines, and plans for decontamination of persons, equipment, and facilities. In response to this directive, the EPA is conducting research to modify and improve efficacy test methods for sporicidal products.

A key priority related to EPA's Homeland Security efforts has been the evaluation and development of quantitative methodology to replace or augment AOAC Method **966.04** Sporicidal Activity of Disinfectants (2), the EPA regulatory standard for performance testing of sporicides. AOAC Method **966.04** is also important to the U.S. Food and Drug Administration's approval process for chemical sterilants. AOAC Method **966.04** provides a qualitative measure of product efficacy against spores of *B. subtilis* and *Clostridium sporogenes* dried on 2 types of carriers, porcelain penicylinders and silk suture loops. Sixty-carrier tests on 3 lots of a product are required for an EPA registration. All carriers must show no growth to support a sporicidal claim. Data generated from AOAC Method **966.04** are more relevant to clinical settings than decontamination scenarios involving buildings and environmental surfaces. As a component of a short-term strategy to address federal regulatory needs, several technical limitations associated with AOAC Method **966.04** have been addressed by EPA (3). The outcome of the effort was the development of a modified and improved version of the method, designated as AOAC Method **966.04**–Method II in the AOAC *Official Methods of Analysis* (OMA; 2). Method I of AOAC Method **966.04** retains unmodified components of the procedure for *Clostridium* and silk suture loops. Compared to the presence/absence outcome associated with qualitative methods such as AOAC Method **966.04**, quantitative methods such as the Three Step Method (TSM) provide a conventional, quantitative measure of efficacy log reduction (LR). LR values may be subjected to statistical analyses to measure the level of confidence in product efficacy, and in the case of a validation study, the LR values and associated variability may be used to determine reliability of the method. Although several well-developed quantitative protocols for sporicides exist in the literature, few have been subjected to multilaboratory validation.

In the precollaborative study associated with this project, 2 quantitative carrier-based test methods for sporicides, the Standard Quantitative Carrier Test Method (QCT-1)–American Society for Testing and Materials (ASTM) E 2111-05 (4) and an adaptation of a quantitative method reported by Sagripanti and Bonifacino (5), were compared across 3 laboratories. The TSM was directly derived from the methodology described in the report by Sagripanti and Bonifacino (5). The goal was to select a single method for use in surrogate studies and eventually validation studies. By conducting the study in a comparative, standardized manner across multiple laboratories, valuable statistical information on the performance of the 2 methods was generated (6). Based on the statistical parameters measured and the additional test method attributes related to logistics and human resources, the TSM was selected for use in *B. anthracis* surrogate studies and was advanced to AOAC validation testing. During the course of the precollaborative studies, EPA refined the TSM for the purpose of surrogate evaluation and the current validation studies. A similar version of the TSM has been accepted and published as a standard procedure by ASTM International; *see* ASTM E 2414-05 (7).

The TSM is a fully quantitative procedure for testing sporicides on hard surfaces. The TSM uses 5 5 1 mm glass coupons (carriers) upon which spores have been inoculated and which are introduced into 400 L liquid sporicidal agent contained in a 1.5 mL microcentrifuge tube. Following exposure to the test chemical and a neutralization agent, spores are removed from carriers in 3 fractions by passive removal (Fraction A), sonication (Fraction B), and gentle agitation (Fraction C). Liquid from each fraction is serially diluted and plated on recovery medium for spore enumeration. Control counts (water control) are compared to the treated counts, and the level of efficacy is the $\log_{10}$ reduction (LR) of spores; LR = mean $\log_{10}$(number of spores/control carrier) – mean $\log_{10}$(number of spores/treated carrier).

The purpose of the collaborative study was to evaluate the reliability of the TSM according to OMA procedures for the purpose of validation. The main statistical goals were to evaluate the repeatability and reproducibility of the LR values, to estimate the components of variance for LR, and to evaluate method responsiveness. AOAC Method **966.04**–Method II was used as a reference method. For the purpose of this study, only the *Bacillus* and hard surface (porcelain penicylinders) components of AOAC Method **966.04**–Method II were included. AOAC Method **966.04**–Method II will be referred to as Method **966.04** for the remainder of this report.

Collaborative Study

Applicability

The scope of the TSM validation was limited to testing liquid formulations against spores of *B. subtilis* (a surrogate for virulent strains of *B. anthracis*) on a hard nonporous surface (glass). The test chemicals used in the study represent 3 different classes of active ingredients: (*1*) sodium hypochlorite (bleach), (*2*) a combination of peracetic acid and hydrogen peroxide, and (*3*) glutaraldehyde. Organic soil was not added to the spore inoculum. AOAC hard water was not used to dilute products; rather, sterile reagent grade water was used as the diluent for diluted products.

Collaborators and Quality Management

Ten laboratories agreed to participate in the collaborative study; collaborators were from government, industry, and contract laboratory sectors. The EPA Office of Pesticide Programs Microbiology Laboratory served as the lead laboratory. 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 level of quality assurance was consistent with EPA Good Laboratory Practices (8). Study protocol familiarization was provided to each laboratory via teleconference before the

study was launched. Each laboratory designated a technical team to conduct the study and was asked to read and practice conducting the test methodologies before the study to gain proficiency.

The Study Director provided the method protocols, standardized data sheets, electronic reporting sheets (MS Excel® format), media preparation sheets, test chemicals, selected media, and reagents. Test chemicals and the Material Safety Data Sheets for each were provided to the collaborators by the Study Director. A single production lot of each test chemical was used in the study. Conditions (e.g., dilution, neutralizer, contact time, and temperature) for testing each test chemical were provided to the laboratories. All manipulations of the test organism were required to be performed in accordance with published biosafety practices (9).

Following data collection, the designated Quality Assurance Officer at each laboratory reviewed and commented on the data and supporting information before submitting the data set to the statistician. Critical findings were immediately communicated to the Study Director.

Study Design

The study design called for 10 laboratories to test 3 chemicals at 3 levels of efficacy (i.e., high, medium, and low) using the TSM, one test chemical at 3 levels/day. Each laboratory conducted 3 independent replicates, one replicate/day, of each test. Assuming no repeat testing, 9 test days were necessary to complete the study. Water controls, used to determine the control carrier counts, were included for each TSM test. Also, each test chemical was evaluated using Method **966.04** to create a reference for the TSM using the same preparations of test chemicals on the first replication only for a total of nine 30-carrier tests per laboratory. The order of testing of chemicals per replication was randomized for each laboratory; however, the order for efficacy level remained the same throughout testing (i.e., high, medium, and low). The TSM control carriers were analyzed last in the sequence on each test day. Also, each laboratory was asked to conduct an HCl resistance test according to the methodology provided in the TSM procedure.

Presque Isle Cultures (Erie, PA) supplied inoculated carriers for the Method **966.04** component. No organic burden was added to the spore inoculum. Before shipping the carriers to the collaborative laboratories, carrier counts and HCl resistance were determined by Presque Isle Cultures and were required to meet the AOAC method specifications. A minimum of 1.0×10^5 spores/carrier [$\log_{10}$ density (LD) = 5.0] and a maximum of approximately 1.0×10^6 spores/carrier were required. Method **966.04** specifies the use of nutrient agar amended with manganese sulfate as the sporulation medium, the same medium used in the TSM. Each laboratory was instructed to perform carrier counts for Method **966.04**. The lead laboratory performed confirmation of neutralizers before the study using the neutralization confirmation procedure identified in the TSM.

Test Chemicals

The test chemicals used in the study were (*1*) sodium hypochlorite (reagent grade, Fisher Scientific Cat. No. SS290-1, sodium hypochlorite solution, 4–6% available chlorine); (*2*) a combination of peracetic acid and hydrogen peroxide (Spor-Klenz Ready to Use, EPA Registration No. 52252-7); and (*3*) 2.6% glutaraldehyde (Metricide 14-Day, a commercially available sterilant). For the purpose of this study, the test chemicals were experimental components only and were not evaluated to verify product label claims. Each chemical was tested at 3 levels of efficacy: high (highly efficacious), medium (intermediate efficacy), and low (nonefficacious). Establishment of treatments with a range of efficacy was important to measure the responsiveness and sensitivity of the TSM. The 3 efficacy levels were selected based on TSM precollaborative data and not on the outcome of Method **966.04**. Because of the instability of test chemicals such as diluted sodium hypochlorite, it was necessary for each laboratory to prepare the actual test formulations on-site. The test conditions used to generate the range of efficacy are shown in Table 1. For sodium hypochlorite, each laboratory prepared the diluted test chemical according to the preparation sheets provided by the Study Director. HACH Test Kits were used to verify available chlorine for diluted sodium hypochlorite samples. Product shelf-life was identified on the preparation sheets. Before the testing, an analysis of formulation chemistry was performed on each lot by EPA to confirm the percent active ingredient. Each product was shown to be within the certified limits applicable to the product. Neutralization confirmation was performed by the lead laboratory before the collaborative study according to Method section **C**(**g**). Luria-Bertani (LB) broth was used to neutralize the peracetic acid/hydrogen peroxide and glutaraldehyde. Sodium thiosulfate (0.1%, w/v) was added to LB broth to neutralizie the sodium hypochlorite. Each neutralizer test chemical combination met the acceptance criteria for neutralization confirmation of 1 log difference in viable spores between neutralizer only and water controls versus the neutralizer/test chemical combination.

Test chemicals were prepared as follows: (*1*) *pH-adjusted sodium hypochlorite.*—Fisher Scientific reagent grade solution diluted to 6000 ± 300 ppm with reagent grade water, pH adjusted (pH 7.0 ± 0.5) with 5% acetic acid, used within ca 120 min after preparation. (*2*) *pH-unadjusted sodium hypochlorite.*—Fisher Scientific reagent grade solution diluted to 6000 ± 300 ppm with reagent grade water, unadjusted pH (pH ~10.0), used within ca 120 min after preparation. (*3*) *pH-unadjusted sodium hypochlorite.*—Fisher Scientific reagent grade solution diluted to 3000 ± 300 ppm with reagent grade water, unadjusted pH (pH ~10.0), used within ca 120 min after preparation. (*4*) *0.08% Peracetic acid/1.0% hydrogen peroxide product.*—Ready-to-use product, used within ca 3 h after dispensing. (*5*) *2.6% Glutaraldehyde product.*—Activated according to product directions, 14 day shelf-life post activation, and used for testing during the 14 day period.

Table 1. Chemical and test condition combinations used for validation of the Three Step Method; 9 total combinations

	Efficacy level and test conditions[a]		
Test chemicals	High (LR 6)	Medium (LR = 2–6)	Low (LR = 0–2)
Sodium hypochlorite (NaOCl)	6000 ± 300 ppm pH adjusted (7 ± 0.5) 30 ± 1 min contact	6000 ± 300 ppm Unadjusted pH (~10) 10 min ± 10 s contact	3000 ± 300 ppm Unadjusted pH (~10) 10 min ± 10 s contact
0.08% Peracetic acid and 1.0% hydrogen peroxide (PA/HP)	30 ± 1 min contact	10 min ± 10 s contact	1 min ± 5 s contact
2.6% Glutaraldehyde	180 ± 3 min contact	60 ± 1 min contact	10 min ± 10 s contact

[a] Test conditions expected to generate 3 levels of efficacy when tested with the TSM; LR = mean $\log_{10}$ reduction in spores.

Test Microbe

The test microbe was *B. subtilis* (ATCC No. 19659). The selection of *B. subtilis* was based on its historical use in Method **966.04** and the laboratory data described below supporting it as a suitable surrogate for *B. anthracis*. Each laboratory was required to either initiate a new stock culture according to the TSM protocol or demonstrate an adequate level of documentation and quality assurance for a pre-existing stock culture generated in accordance with the TSM procedure. Spores for inoculation of TSM carriers were produced by each collaborating laboratory according to the TSM protocol. The mean target carrier load for the TSM was 1.0 10^7 spores/carrier or 7.0 logs/carrier—a level suitable for measuring an LR of 6. Mean carrier counts within a range of 5.0 10^6–5.0 10^7 spores/carrier were permissible. No organic burden was added to the spore inoculum. TSM carriers were inoculated from one spore preparation per laboratory; a suitable number of carriers were inoculated as a set to perform the entire study (approximately 150).

B. subtilis as a Surrogate of B. anthracis

Because of the Homeland Security implications associated with this project, a prevalidation study (data unpublished) was conducted by EPA in advance of the TSM validation to select a relevant test microbe, i.e., a surrogate of *B. anthracis*. The health and safety requirements for handling and testing virulent *B. anthracis* are difficult to satisfy for most laboratories, and without a surrogate, efficacy testing of virulent *B. anthracis* will be limited to a few laboratories. To be an acceptable surrogate, a *Bacillus* spore species should generally be as resistant or more resistant to inactivation by a particular chemical on a particular surface than are *B. anthracis* spores. In the prevalidation study, the resistance of spores of *B. subtilis* (ATCC No. 19659), *B. anthracis*–Ames (virulent strain) and *B. anthracis*– Sterne (avirulent strain) against liquid sporicides on a hard nonporous surface (glass) using the TSM was compared. High-quality spore preparations of each microbe were successfully generated according to the TSM procedure. Three test chemical treatments were used: (*1*) 3000 ppm sodium hypochlorite with adjusted pH (~7.0); (*2*) 3000 ppm sodium hypochlorite with unadjusted pH (~10.0); and (*3*) a peracetic acid (0.08%) and hydrogen peroxide (1.0%) product. Sodium hypochlorite with unadjusted pH was used as an intermediate efficacy treatment. Glutaraldehyde was not included in the study because of its limited use in treating surfaces contaminated with spores of *B. anthracis*. Inoculated carriers were exposed to the test chemicals for 10 min. Three replications (days) for each of 3 chemicals were performed. The acceptable range for spore load per control carrier was 5.0 10^6–5.0 10^7. Neutralization was achieved using chilled LB broth. Sodium thiosulfate (0.1%, w/v) was added to LB broth to neutralize the sodium hypochlorite. Control counts for each microbe were comparable; mean LD of spores per carrier were 7.1, 7.1, and 7.2 for *B. subtilis*, *B. anthracis*–Ames, and *B. anthracis*– Sterne, respectively. Equivalence testing showed the control means for all pairs of microbes were statistically equivalent. Overall, the LR values for *B. subtilis* spores were always numerically equal to or lower than *B. anthracis*– Sterne, and in 7 of the 9 experiments, *B. subtilis* exhibited lower LR values than *B. anthracis*–Ames. With only one exception, LR values for *B. subtilis* across each chemical treatment were not statistically significantly different from *B. anthracis*–Ames or *B. anthracis*– Sterne at the 0.05 significance level. The exception was that *B. subtilis* exhibited significantly lower LR than *B. anthracis*–Ames for sodium hypochlorite with unadjusted pH (paired *t*-test p = 0.04). *See* Table 2 for a summary of the data. Using the TSM, *B. subtilis* was shown to be at least as resistant or more resistant to the test chemicals than either *B. anthracis* strains when tested on a hard nonporous surface, thus providing support for its use as a relevant surrogate in the method validation. The conclusions are limited to the liquid test chemicals and coupon materials used in the prevalidation study. We recognize that other *Bacillus* species (e.g., *atrophaeus, cereus, stearothermophilus*) may be more appropriate surrogates for *B. anthracis* when other chemical agents and formulations are evaluated on different coupon materials (e.g., porous materials), and that surrogate selection may require comparative studies on a case-by-case basis.

Table 2. Comparative log reduction data for *Bacillus anthracis* surrogate study

Microbes (spores)	Log reduction values (with S_r)/test chemical[a]		
	Sodium hypochlorite with unadjusted pH	Sodium hypochlorite with adjusted pH	Peracetic acid and hydrogen peroxide
Bacillus subtilis	1.3 (0.66)	4.9 (0.71)	5.5 (0.18)
Bacillus anthracis–Ames	4.5 (0.91)	5.8 (0.92)	5.1 (1.00)
Bacillus anthracis– Sterne	4.6 (1.18)	6.1 (0.28)	5.9 (0.53)

[a] S_r = Repeatability standard deviation.

Statistical Analysis General Methodology

In quantitative studies such as this, it is not unusual for a dilution series to miss the countable range of viable cells, thereby providing anomalous counts, either all zeros or all "too numerous to count" (TNTC). For such anomalous data, artificial counts were substituted to facilitate statistical analysis. If all dilutions of a fraction (A, B, or C) produced TNTC, 300 was substituted at the last dilution for that TSM fraction. If all dilutions of all 3 fractions produced zero counts, 0.5 was substituted at the first dilution of Fraction A.

The spore count for each TSM carrier was the sum of the counts in the separate Fractions A–C. To reveal the relative contributions of the individual fractions, the percentages of the total spore counts attributable to each fraction were calculated.

For both TSM and Method **966.04**, each control carrier spore count was $\log_{10}$–transformed to create the LD. The statistical properties of the control carrier LDs were calculated using analysis of variance (ANOVA) techniques.

For the TSM, the LR was calculated for each test by taking the mean of LDs for control carriers minus the mean of densities for treated carriers (10). For Method **966.04**, the LR was calculated using the positive/negative (P/N) formula (3). Because Method **966.04** was modified to require the enumeration of spores on control carriers, the P/N formula could be applied to increase the utility of Method **966.04** as a reference method for the TSM. For each method and for each combination of test chemical and level of efficacy, the statistical properties of LR were calculated by ANOVA. Plots of mean LR versus efficacy were used to show whether the mean LR values correctly ordered the test chemicals of low, medium, and high efficacy.

The ANOVA models required the assumption that the collaborating laboratories were statistically representative of all laboratories that will conduct the TSM. Statistical calculations were performed using 2 statistical software products, the nlme package in R (R—Software Environment for Statistical Computing and Graphics, http://www.r-project.org/) and Minitab (Release 14, Minitab Statistical Software, http://www.minitab.com/products).

It is desirable for the repeatability standard deviation (S_r) and reproducibility standard deviation (S_R) to be low. For quantitative antimicrobial product tests, AOAC INTERNATIONAL has no specifications for concluding that a standard deviation (SD) is acceptably low. Some guidance is provided by a recent literature review which showed that, for established suspension and dried surface antimicrobial product tests, S_r ranged from 0.2 to 1.2 with a median of 0.4 and S_R ranged from 0.3 to 1.5 with a median of 0.8 (11). It would be reasonable to claim that the S_r and S_R are acceptably low if they fall within these ranges.

The bias of LR cannot be assessed for these sporicidal test methods because of the unavailability of an accepted "true numerical value" for LR. It may be reasonable, however, to speculate about potential bias if anomalous LR trends are observed. Because of the nature of sporicidal testing, the conceptual, unknown, true LR is specific to the test method. It is implausible that the true LR for a TSM test of a test chemical is identical to that for a Method **966.04** test of the same test chemical.

Statistical Analysis ANOVA Models

For the analysis of LDs on TSM control carriers, the ANOVA was based on a nested, 2-factor, random effects model (12). The ANOVA provided the numerical estimates: S_L^2, which denotes the variance among laboratories; S_T^2, which denotes the variance among independent tests within a laboratory; and S^2, which denotes the variance among carriers within a test. Let $S_R^2 = S_L^2 + S_T^2 + S^2$ denote the "total variance." Then $S_R = \sqrt{S_R^2}$ is the reproducibility standard deviation and $S_r = [S_T^2 + S^2]^{1/2}$ is the repeatability standard deviation (13). The standard error of the mean (SEM), which is the square root of the variance of the mean LD, was calculated using the formula (14): $SEM = \{[S^2/(3 \cdot 9 \cdot 8)] + [S_T^2/(9 \cdot 8)] + [S_L^2/8]\}^{1/2}$. In the SEM formula, 3 = 3 control carriers observed on each test day in each laboratory, 9 = 9 test days (3 chemicals each tested on 3 days), and 8 = 8 laboratories.

Each of the 8 laboratories recorded spore counts on each of 5 Method **966.04** control carriers, thereby producing 8 mean LD values. The SD of those 8 means is S_R, the reproducibility standard deviation for the mean of 5 control carrier LDs. The statistical summary included the mean and SEM of the 8 laboratory means, where $SEM = S_R/\sqrt{8}$.

For each combination of test chemical and level of efficacy, the LR values for the TSM were submitted to ANOVA based on a one-way, random effects linear statistical model. Let $S_R^2 = S_L^2 + S_T^2$ denote the total variance, where S_L^2 denotes the variance of LR among laboratories, and S_T^2 denotes the

within-laboratory variance of LR. Then $S_R = \sqrt{S_R^2}$ is the reproducibility standard deviation and $S_r = \sqrt{S_T^2}$ is the repeatability standard deviation (13, 15). The ANOVA provided numerical estimates: S_L^2, S_T^2, S_r, and S_R. For each test chemical and level of efficacy, the formula for the SEM of the mean LR was (12): SEM = $\{[S_T^2/(3\cdot8)] + [S_L^2/8]\}^{1/2}$. For each treatment, the mean LR is an estimate of the conceptual true LR for that treatment, and the mean LR ± t·SEM, where t denotes the appropriate quantile of the t probability distribution based on 7 degrees of freedom, provides the endpoints of a confidence interval estimate of the true LR. For example, mean LR ± 1.00·SEM provides a 65% confidence interval and mean LR ± 2.36·SEM provides a 95% confidence interval for the true LR for the treatment.

The LR values for Method **966.04** were calculated using the P/N formula (3). Because the Method **966.04** tests were not replicated within laboratories, there was a single LR for each test chemical and level of efficacy that was evaluated and a single mean of 5 control carriers in each of the 8 laboratories. The SD of the 8 values was the S_R. The quantities S_L^2, S_T^2, and S_r were incalculable. For each test chemical and level of efficacy, the SEM of the mean LR was SEM = $[S_R^2/8]^{1/2}$.

AOAC Official Method 2008.05
Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard Nonporous Surface

Quantitative Three Step Method
First Action 2008

(Applicable for determination of sporicidal activity of liquid formulations against spores of the genus *Bacillus* on a hard nonporous surface. The method is suitable for testing strains of *B. anthracis.*)

Caution: All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated by each institution. Use the equipment and facilities indicated for the test organism. For recommendations on safe handling of microorganisms, refer to the Centers for Disease Control and Prevention/National Institutes of Health (CDC/NIH) Biosafety in Microbiological and Biomedical Laboratories (BMBL) manual (1). *B. anthracis* is a select agent and therefore appropriate regulations must be followed (*see* BMBL Appendix F). Sporicidal products may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Also, hydrochloric acid (HCl) is a highly corrosive liquid and considered hazardous. Personal protective equipment (PPE) or devices are recommended during the handling of these items for purpose of activation, dilution, or efficacy testing. PPE and a chemical fume hood should be used when performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine best course of action.

Note: References to water indicate reagent grade water, except where otherwise specified (2). Exact adherence to the method, good laboratory practices, and quality control (QC) are required for proficiency and validity of the results.

A. Media and Reagents

(**a**) *Media.*—(*1*) *Nutrient broth (NB).*—Dehydrated. For use in rehydrating test organism and preparing nutrient agar.

(*2*) *Nutrient agar (NA).*—For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized NB. Boil mixture until agar is dissolved. If necessary, adjust pH to 7.2 ± 0.2. 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. Dehydrated NA may be substituted; suspend 23 g NA/L water and dissolve by boiling. If necessary, adjust pH to 6.8 ± 0.2. Autoclave for 15 min at 121 C.

(*3*) *NA with 5 μg/mL manganese sulfate monohydrate ($MnSO_4$ H_2O) (amended NA).*—For spore production. Suspend 11.5 g NA in 495 mL water and add 5 mL 500 ppm $MnSO_4$ H_2O. Dissolve by boiling. If necessary, adjust pH to 6.8 ± 0.2. Autoclave for 15 min at 121 C. Pour agar into plates.

(*4*) *Trypticase soy agar (TSA).*—Poured in plates for microbe isolation and spread plating.

(*5*) *Luria-Bertani (LB) broth.*—Dehydrated (e.g., Difco); suspend 25 g LB broth in 1 L water, mix well, if necessary adjust pH to 7.0 ± 0.2, dispense in bottles, and autoclave for 15 min at 121 C; use as neutralizer.

(*6*) *Modified LB broth.*—Neutralizer in HCl resistance test. Add 20 mL 1 M NaOH to 1 L LB broth, mix well, dispense in bottles, and autoclave for 15 min at 121 C.

(*7*) *LB broth with 0.1% (w/v) sodium thiosulfate.*—Neutralizer for sodium hypochlorite treatments. Add 1.0 g sodium thiosulfate to 1 L LB broth, mix well, dispense in bottles, and autoclave for 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**) *Sodium thiosulfate.*

(**d**) *Water.*—Sterile, reagent grade, 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.

(**e**) *Test organism.—B. subtilis* (ATCC No. 19659) obtained directly from a commercial supplier (e.g., American Type Culture Collection, Manassas, VA).

B. Apparatus

(**a**) *Certified biosafety cabinet (Class II).*—Recommended to maintain an aseptic work environment.

(**b**) *Glass coupon*.—Hard surface carrier, 5 5 1 mm (Erie Scientific Co., Portsmouth, NH); custom order Part No. EPA-1101 (minimum order of 1000 pieces), single use.

(**c**) *Microcentrifuge tubes*.—Sterile, 1.5 mL (Fisher Scientific, Waltham, MA; Cat. No. 05-408-129).

(**d**) *Centrifuge tubes*.—Sterile, polypropylene, 15 mL conical tubes with conical bottoms (Fisher Scientific; Cat. No. 05-538-53D).

(**e**) *Dissecting forceps*.—VWR Cat. No. 25607-195 (West Chester, PA) or Fisher Scientific Cat. No. 13-812-42.

(**f**) *Micropipet*.—Calibrated.

(**g**) *Positive displacement pipet*.

(**h**) *Desiccator.*

(**i**) *Water bath/chiller unit*.—Constant temperature, capable of maintaining 20 1 C temperature or specified temperature; e.g., Neslab RTE-221 (Waltham, MA) or Nalgene Labtop Cooler (Rochester, NY).

(**j**) *Orbital shaker.*

(**k**) *Microcentrifuge*.

(**l**) *Microcentrifuge tube lid openers*.—USA Scientific No. 1400-1508 (Ocala, FL).

(**m**) *Sonicator.*—Ultrasonic cleaner (Branson Model 1510 Bath Sonicator, Danbury, CT; or equivalent).

(**n**) *Floating microcentrifuge tube holder.*—For sonication (VWR No. 60986-099).

(**o**) *Hematology rotator.*—Hematology Chemistry Mixer 346 (Fisher Scientific); or a suitable mixer/shaker to provide gentle agitation during incubation.

(**p**) *Vortex mixer.*

(**q**) *Vortex adapters*.—Fisher Scientific Cat. Nos. 1281161 and 1281211.

(**r**) *Certified timer.*—Any certified timer that can display time in seconds.

(**s**) *Test tubes*.—25 150 mm.

(**t**) *Ethyl alcohol*.—40 and 95%.

C. Operating Technique

(**a**) *Culture initiation*.—Initiate *B. subtilis* culture (e.g., use NB to rehydrate a lyophilized culture, and incubate broth culture for 24 2 h at 36 1 C prior to streak inoculation). Streak inoculate a set (e.g., 6) NA slopes and incubate 24 2 h at 36 1 C. Perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or other identification systems). Following incubation, store at 2–5 C. Maintain stock culture on NA 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 NB and incubate tubes 24 2 h on an orbital shaker at approximately 150 rpm at 36 1 C. Use this culture to inoculate amended NA plates. Inoculate each plate with 500 L broth culture and spread inoculum with 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. Following incubation, harvest the spores by adding 10 mL cold (2–5 C) 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, ~10 mL each). Centrifuge tubes at 5000 rpm for approximately 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 10 1 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). Spores versus vegetative cells should be at least 95%. Spore suspension harvested from multiple plates can be combined and re-aliquoted into tubes for uniformity. Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0 10^{-6}–1.0 10^{-8}) onto TSA. Incubate plates for 24 2 h at 36 1 C and determine titer. *Note*: When harvested and processed, 10 plates of amended NA should provide 80–100 mL concentrated spore suspension. Diluting the suspension prior to carrier inoculation will be necessary; a spore titer of approximately 1.0 10^9 colony-forming units (CFU)/mL in the suspension should be adequate to achieve the target carrier count.

(**c**) *Carrier preparation.*—Visually screen glass coupons (carriers) for scratches, chips, or cracks. Discard those which are damaged or defective. Rinse carriers once with water, 3 times with 95% ethyl alcohol, and finally 3 times with water. Allow carriers to dry. Place in glass tubes (25 150 mm), 40 carriers per tube. Steam-sterilize 45 min at 121 C with a 30 min dry cycle or sterilize for 2 h in hot air oven at 180 C. Cool. Transfer carriers to sterile plastic Petri dishes for inoculation (approximately 40 carriers per dish).

(**d**) *Carrier inoculation*.—Transfer 10 L spore suspension with a micropipet using aerosol barrier tips or positive displacement pipet onto a 5 5 1 mm sterile, dry glass coupon. Apply to one central spot on each carrier. Allow carriers to dry for minimum of 1 h in open Petri dish in a biosafety cabinet, then for a minimum of 12 2 h in a desiccator. Store inoculated carriers under desiccation for up to 30 days. Glass carriers must be discarded after use. *Note*: During carrier inoculation, mix inoculum frequently in Vortex mixer to ensure uniform distribution of spores. Verify carrier counts (according to the method for control carriers) prior to test day; mean counts must be 5.0 10^6 to 5.0 10^7 spores/carrier. *Note*: Because of the occurrence of statistical variability in the log reduction (LR) data, it is recommended that the analyst target carrier counts of 7–7.5 logs to ensure confidence in a 6 LR.

(**e**) *Test chemical (e.g., sporicide, disinfectant) sample preparation*.—Aseptically prepare test chemical samples as directed. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use 1.0 mL or 1.0 g sample test chemical to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Place approximately 1.5 mL of each test chemical

or control (sterile water) in microcentrifuge tubes. Allow to equilibrate to appropriate temperature for 15–30 min.

(**f**) *Test procedure overview.*—A minimum of 3 carriers per test chemical and 3 carriers for the water control (control carriers) are required per product test. Use one pair of sterile forceps per fraction for each test chemical. Fractions may be refrigerated briefly to allow for processing of other fractions. It is recommended that 2 analysts perform this method so that dilution and plating of the multiple fractions may be conducted as soon as possible.

Using sterile forceps, carefully transfer one inoculated carrier into each microcentrifuge tube labeled Fraction A. Avoid touching inoculated area of carrier and sides of microcentrifuge tube. Discard carrier and tube if carrier touches sides of tube. Place Fraction A tubes containing carriers and tubes containing test chemical(s) and sterile water (control) into chiller water bath at 20 1 C, or use a labtop cooler to maintain temperature of the tubes. Equilibrate approximately 10 min. Add 400 L test chemical (test carriers) or 400 L sterile water (control carriers) at 15 or 30 5 s intervals to appropriate microcentrifuge tube (in triplicate). Allow contact of the carriers to the test chemical or water in Fraction A tubes for the appropriate exposure period.

Following the exposure period, add 600 L of appropriate ice-cold neutralizer (e.g., LB broth) to each test chemical Fraction A tube. Add 600 L LB broth as neutralizer for water control Fraction A tubes. Slightly agitate tubes to thoroughly mix liquid components. Transfer each carrier using one pair of sterile forceps per carrier set (i.e., 3 carriers) from Fraction A tube to corresponding Fraction B tube. Fraction B tubes contain 400 L ice-cold (0–5 C) sterile water.

Place Fraction A tubes in microcentrifuge, centrifuge for 6 min 30 s at 13 000 rpm (15 500 *g*). Remove 900 L from each tube without disturbing pellet. Discard supernatant. Carefully add 900 L ice-cold LB broth to each tube. Repeat 2 additional times. After third centrifugation, remove 900 L from each tube. Carefully add 100 L ice-cold LB broth to each Fraction A tube and resuspend pellet by mixing in a Vortex mixer 5 min 30 s (use the Vortex adapter) at midrange speed. Add 800 L ice-cold LB broth to each Fraction A tube. Proceed to dilution and plating if another analyst is available, or store Fraction A tubes in refrigerator. *Note*: Fluid remaining in the Fraction A tubes contains spores dislodged from carrier by exposure to the test chemical or water control. Consistent orientation of the microcentrifuge tubes in the microcentrifuge is important in locating the pellet. The pellet may range in size and be difficult to visualize depending on the treatment. Fractions B and C tubes can be evaluated while Fraction A tubes are being centrifuged.

Sonicate Fraction B tubes 5 min 30 s using a floating microcentrifuge tube holder placed inside an ultrasonic cleaner. After sonication is complete, add 600 L ice-cold LB broth to Fraction B tubes. Mix on a Vortex mixer approximately 1 min. Transfer each carrier using one pair of sterile forceps per carrier set from Fraction B tube to corresponding Fraction C tube (Fraction C tubes contain 400 L ice-cold LB broth). Proceed to dilution and plating if another analyst is available, or store Fraction B tubes at 2–5 C; storage should be limited to 2 h. *Note*: Fluid remaining in the Fraction B tubes contains spores dislodged from the carrier by sonication.

Place Fraction C tubes in a hematology rotator inside incubator for 30 2 min at 36 1 C. Remove Fraction C tubes after 30 2 min rotation/incubation from incubator. Add 600 L ice-cold LB broth to each tube. The carriers remain in the Fraction C tubes. Proceed to dilution and plating if another analyst is available, or store Fraction C tubes at 2–5 C; storage should be limited to 2 h. *Note*: Fluid remaining in Fraction C tubes contains spores dislodged from the carrier by gentle agitation for 30 min.

Mix on a Vortex mixer each microcentrifuge tube thoroughly prior to making dilutions. For each fraction and control tube, remove 100 L and serially dilute 10-fold in 900 L ice-cold LB broth. For each carrier, direct plate 100 L of the sufficient dilutions onto TSA to ensure obtaining counts within the target range of 30–300 CFU/plate. Incubate plates a minimum of 24 2 h at 36 1 C. Record control counts at 24 2 h. Record treated carrier counts at 24 2 and at 48 2 h. Confirm the identity of a minimum of one representative colony taken from at least one plate per treatment level (if available) using Gram staining, general growth media (e.g., TSA), or other confirmation procedure. *B. subtilis* is a large Gram-positive rod. On general growth media, *B. subtilis* colonies are opaque, rough, round, low convex colonies with irregular margins. *Note*: After plating, dilution tubes may be stored at 2–5 C until the results are recorded; the tubes may be used for additional plating if initial plate counts are beyond the recommended target range.

Use counts that fall within 0–300 CFU/plate for calculations. Obtain the total number of spores per fraction by dividing the number of colonies counted in each fraction by its dilution, and account for volume plated. Obtain the total number of spores per carrier by adding the total number of viable spores per fraction for Fractions A–C. Determine log density (LD) of total number of viable spores per carrier by taking $\log_{10}$ (total number of spores per carrier). Determine LR by subtracting the mean LD of test carriers from the mean LD of control carriers. Determine average LD and LR for each test chemical.

(**g**) *Neutralization confirmation.*—Use 12 microcentrifuge tubes. Add 400 L sterile water to tubes 1–6 and 400 L test chemical to tubes 7–12. Allow tubes to equilibrate approximately 10 min at 20 1 C (or other specified temperature). Add 600 L neutralizer in ice-cold LB broth (or only LB broth depending on the product) to tubes 4–6 (neutralizer controls). Add 600 L neutralizer in ice-cold LB broth to tubes 7–9 (ability of neutralizer to inactivate the test chemical). Gently mix. Add 10 L *B. subtilis* spore suspension (approximately 10^9 spores/mL) to each tube and mix in a Vortex mixer for approximately 15 s. Incubate tubes for 30 2 min at 20 1 C (or temperature specified by test chemical manufacturer). After incubation, add 600 L ice-cold LB broth to tubes 1–3 (survival controls). Add 600 L ice-cold LB broth to tubes 10–12 (test chemical

controls). Serially dilute each tube (e.g., 10 L into 990 L ice-cold LB broth or 100 L into 900 L ice-cold LB broth) to achieve plate counts of 30–300 CFU/plate. Plate 100 L of each dilution onto TSA. Incubate 24 2 h at 36 1 C. Count colonies on each plate. LD (CFU/mL) in tubes 1–3 and 4–6 should reflect the original spore suspension titer and should be within 1 log of each other. If the difference in LD between tubes 1–3 and 4–6 is greater than 1 log, then the neutralizer has a sporicidal effect. If the test chemical is highly effective, LD in tubes 10–12 should be approximately 5–6 logs lower than LD in tubes 1–6. To be an effective neutralizer, LD in tubes 7–9 should be within 1 log of the LD in tubes 1–6. For this assay, produce a spore preparation according to the procedure for amended NA. Harvest growth from plates (e.g., 5 plates) according to the method, except resuspend pellet after final centrifugation step in approximately 100 mL aqueous (40%) ethanol.

(**h**) *HCl resistance*.—Perform on each preparation of inoculated carriers. Conduct TSM procedure on 2.5 M HCl. Follow procedure as specified in (**f**) with 2 and 5 min exposure periods with 3 inoculated carriers per time period. Include 3 control (sterile water) carriers to determine control carrier counts. Use LB broth modified with NaOH as the neutralizer instead of LB broth for HCl treatments. Perform test at 20 1 C. Calculate LR. Spores should resist HCl for 2 min (i.e., based on presence of viable spores after 2 min) to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described. *Note*: Compared to the water control, anticipate LR of 0–3 at 2 min exposure and LR of 2–6 following the 5 min exposure.

References: (1) *Biosafety in Microbiological and Biomedical Laboratories (BMBL)* (2007) 5th Ed., Centers for Disease Control and Prevention and National Institutes of Health, Washington, DC.

(2) *Standard Methods for the Examination of Water and Wastewater* (2005) 21st Ed., American Public Health Association, Washington, DC.

(3) *J. AOAC Int*. **91**, 833(2008).

Results and Discussion

The TSM validation data were collected over a 4 month period. Peer review of the data entry was performed by laboratory personnel prior to submitting the data set to the statisticians. Data from only 8 of the 10 participating laboratories were used in the statistical analysis because one laboratory did not generate data in the established timeframe and one laboratory significantly deviated from the test protocol for preparation of the spore inoculum. Otherwise, all aspects of the experimental design were carried out according to the collaborative study protocol.

All data were deemed valid by the Study Director and were included in the analysis. If an unusual observation was discovered during the quality assurance review or the statistical analysis, the raw data entry form was consulted. If questions remained, the data were discussed with the appropriate laboratory personnel. No data errors were discovered.

*Method **966.04***

There was a limited amount of data on the performance of quantitative antimicrobial product test methods, and consequently, few quantitative methods were available for use as true reference methods. Despite its qualitative nature, Method **966.04** was selected as the reference method because of its extensive historical use as a regulatory method. As the reference method, Method **966.04** provided comparative baseline efficacy data for the 9 test chemical and level of efficacy combinations used in the study. Method **966.04** was conducted a total of 72 times; 8 laboratories tested 3 test chemicals at 3 efficacy levels, one replication per combination. The inclusion of Method **966.04** in this study proved valuable in the overall validation scheme. The raw data (e.g., control counts, number of positive carriers) for AOAC Method **966.04** are presented in *Appendix A*.

Control counts.—Forty control carriers were analyzed (5 carriers per laboratory) for spore titer. The mean of control

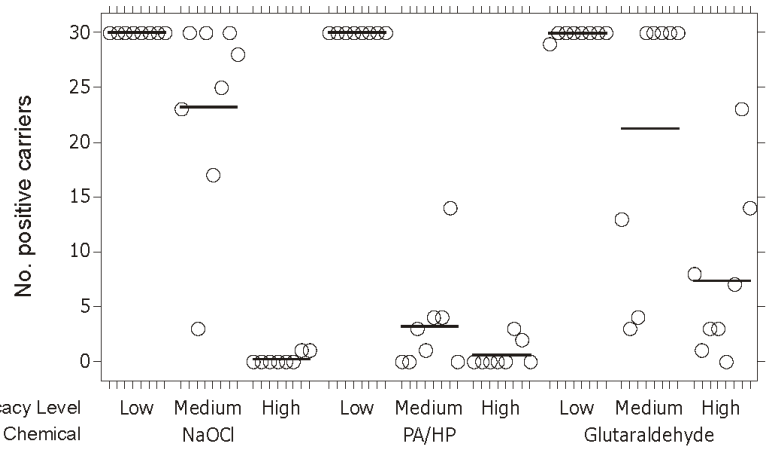

Figure 1. Each symbol denotes the number of positive carriers in one laboratory for AOAC Method 966.04 using 30 carriers. For each test chemical, the 8 symbols are arranged from left to right by increasing laboratory number. Each horizontal line indicates the mean, averaged over the 8 laboratories, for the test chemical treatment.

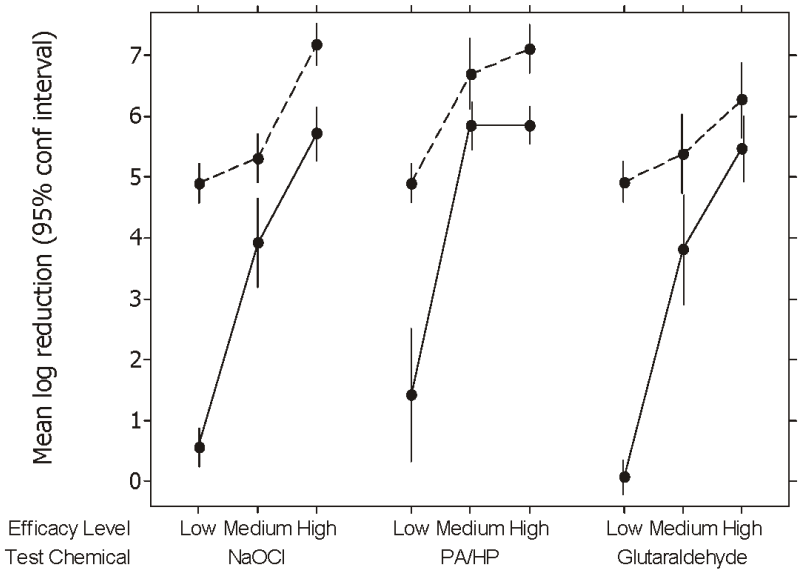

Figure 2. Log reduction versus efficacy level for each test chemical plotted for AOAC Method 966.04 (dashed lines) and the TSM (solid lines). Each symbol indicates the mean LR, averaged across laboratories, and the error bar is the 95% confidence interval.

carrier LDs fell within the prescribed range of 5 to approximately 6 logs spores per carrier in 7 of 8 laboratories; the range was 4.9 to 5.9 logs per carrier. Across laboratories, the mean log spore density per carrier (±SEM) was 5.5 (±0.13). The S_R for the mean of 5 control carriers was 0.38. The mean LD per carrier generated by the vendor was 5.9 (data provided to the Study Director); this value was based on 5 carriers analyzed prior to shipment using the enumeration procedure described in Method **966.04**. For the purpose of this investigation, the laboratory with the mean of 4.9 logs spores per carrier was not excluded from the analysis. Slight differences in storage, sonication time, and the type of ultrasonic cleaner used in the enumeration may account for the 1 log range in the carrier counts. Also, one laboratory noted that the type of beaker used to hold the polypropylene tube containing the porcelain carrier in the ultrasonic cleaner was not specified (plastic versus glass) in the protocol. This may be another factor worthy of investigation in order to further standardize Method **966.04**. Despite the slightly low LD value from one laboratory, the LD values observed for Method **966.04** are consistent with the collaborative study involving the modification of Method **966.04** and the associated use of amended nutrient agar as the sporulation medium (3).

Qualitative efficacy.—Method **966.04** effectively distinguished the highly efficacious formulations from the low nonefficacious formulations, and adequately served as a reference method for the validation study. *See* Figure 1 for the number of carriers that exhibited growth per each test chemical treatment. Method **966.04** accurately ranked efficacy for 8 of 9 test chemical treatments across laboratories; the only exception was the medium peracetic acid/hydrogen

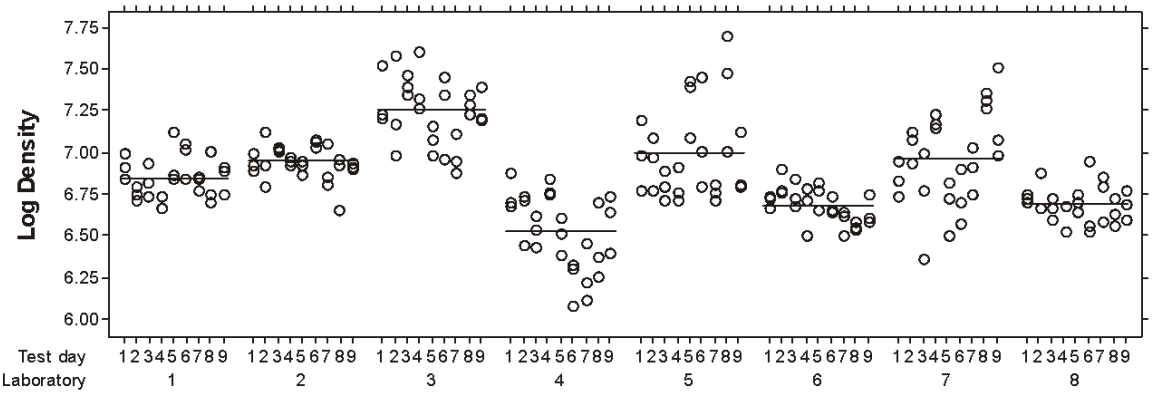

Figure 3. Each symbol is one TSM control carrier. The $\log_{10}$ densities for the 3 control carriers on each day in each laboratory are aligned vertically. The test days are numbered in chronological order within each laboratory. The horizontal lines show the mean log densities for the laboratories.

Table 3. Summary of TSM HCl resistance data for *Bacillus subtilis* spores; laboratory procedure described in C(h)

		Log density for 3 carriers		LR/exposure time	
Aspect	Control	2 min	5 min	2 min	5 min
Mean	6.93	5.14	3.01	1.80	3.93
Min.	6.64	3.86	1.89	0.23	2.06
Max.	7.37	6.40	4.58	2.92	5.41
S_R	0.28	0.81	0.90	0.99	1.03

peroxide (PA/HP), which exhibited similar efficacy as the high level. The mean number of positive carriers was 3.2 and 0.6 out of 30 for the medium and high PA/HP levels, respectively. For the PA/HP, the conditions for the high and medium efficacy levels were different by contact time, 30 and 10 min, respectively. A 1 min contact time was used to establish the low efficacy levels for PA/HP, resulting in all positive carriers across the laboratories. Precollaborative studies indicated a mean LR of 4.6 for PA/HP at 10 min with the TSM (data not shown). The similar efficacy measured for the medium and high PA/HP levels is more indicative of the nonlinear nature of product activity and not an indicator of limited sensitivity for Method **966.04**. As discussed below, results for Method **966.04** and the TSM tracked similarly for the medium and high PA/HP levels.

Thirteen of the 24 tests of the high efficacy test chemical treatments resulted in complete kill (0/30 carriers positive); the high glutaraldehyde treatment displayed the greatest variability with a range of 0–23 positive carriers. The mean number of positive carriers for the high sodium hypochlorite, PA/HP, and glutaraldehyde treatments were 0.3, 0.6, and 7.4 out of 30 total carriers, respectively. For the 24 tests of the 3 low efficacy test chemical treatments, 23 exhibited all positive carriers. The performance results from the 24 tests for the 3 medium efficacy test chemical treatments were more variable than the high and low levels—a range of 0–30 carriers with growth was observed across the medium efficacy test chemistry treatments.

Log reduction calculations.—To further aid in the comparative process, the P/N formula (3) was used to estimate LR values for Method **966.04**. The LR in spores per carrier for each test chemical increased with increasing efficacy level (Figure 2), as expected. Across all test chemical treatments, the LR values ranged from 4.90 to 7.18. S_R was estimated for only those treatments where there was interlaboratory variability in the number of positive treated carriers. Specifically, the S_R estimate was based on only 4 test chemical treatments for which 3 or more laboratories found 1 to 29 positive carriers out of 30 (medium sodium hypochlorite, medium PA/HP, and medium and high glutaraldehyde). The LR values for those 4 treatments produced S_R = 0.69, an acceptably low value. Based on the P/N procedure, Method **966.04** was unable to discriminate LR values <5. Although the mean number of positive carriers in the 3 low test chemical treatments ranged from 0.3 to 7.4 out of 30, the LR values generated using the P/N formula for each were approximately 4.9. An accurate assessment of LR for the low test chemical treatments was problematic using the P/N formula, i.e., the low treatments were mainly represented by treatments with 30 out of 30 positives, thus reducing the sensitivity of the P/N procedure to measure the LR.

Three Step Method

Control counts.—The TSM control counts for each laboratory are presented in *Appendix B*. Each laboratory independently prepared a *B. subtilis* spore suspension for use in the TSM; thus, some degree of variation in the control

Table 4. Mean LR values, variance components, and the repeatability and reproducibility standard deviations presented for each of the 9 test chemical treatments based on 3 replicate tests in each of 8 laboratories; S_T^2 is the variance among independent tests within a laboratory and S_L^2 is the variance among laboratories

				95% confidence limits for LR		Variance components			
Test chemical	Efficacy level	Mean LR	SEM	Lower	Upper	Within labs (S_T^2)	Among labs (S_L^2)	S_r	S_R
NaOCl	Low	0.56	0.13	0.24	0.87	0.1641	0.0874	0.41	0.50
NaOCl	Medium	3.92	0.31	3.19	4.65	0.2008	0.7004	0.45	0.95
NaOCl	High	5.71	0.18	5.29	6.14	0.2645	0.1703	0.51	0.66
PA/HP	Low	1.41	0.46	0.32	2.51	0.4915	1.5582	0.70	1.43
PA/HP	Medium	5.85	0.16	5.47	6.22	0.3334	0.0898	0.58	0.65
PA/HP	High	5.85	0.13	5.54	6.16	0.4083	0.0000	0.64	0.64
Glutaraldehyde	Low	0.07	0.11	–0.19	0.34	0.0293	0.0894	0.17	0.34
Glutaraldehyde	Medium	3.81	0.38	2.91	4.72	0.5172	0.9964	0.72	1.23
Glutaraldehyde	High	5.47	0.22	4.94	6.00	0.2275	0.3276	0.48	0.75

counts was anticipated from laboratory to laboratory. The TSM protocol allows for dilution of the concentrated spore inoculum as necessary to achieve the target carrier counts of 5 10^6–5 10^7 mean spores per carrier (geometric mean) or 6.7–7.7 logs per carrier. Across all laboratories, the mean LDs per test ranged from 6.2 to 7.4 (Figure 3). For the 72 total TSM tests, the mean (±SEM) LD of spores per carrier was 6.86 (±0.08), where the SEM is based on the ANOVA component estimates, $S^2 = 0.021$, $S_T^2 = 0.016$, and $S_L^2 = 0.049$. Seventy-four percent of the mean LDs, averaged over the 3 control carriers per test day, fell within the proposed target range and none were 7.7. For the mean of 3 control carrier LDs, S_r and S_R were low at 0.15 and 0.27, respectively. In the prevalidation study, the TSM mean control LD per carrier was 7.5, $S_r = 0.18$, and $S_R = 0.18$. In this study, the slightly higher S_R was due to a greater among-laboratory variance that comprised 57% of the total variance. The TSM LR calculations were based on the difference between mean LDs, control minus treated, within each individual test; thus, the among-laboratory variance of the control densities did not affect the statistical properties of the LR values. Meeting the target carrier count range specified in the TSM was shown to be very feasible; however, based on the data, a slight change in the final TSM protocol to dilute the spore suspension less to achieve a titer closer to 5 10^9 rather than 1 10^9 spores/mL is recommended. The adjustment will provide carrier counts at or slightly above 7 logs of spores/carrier; 7 logs is necessary to effectively measure an LR of 6.

Percent recovery of spores from inoculated TSM glass carriers has historically been very high (90%). Although not a requirement for validation, the percent recovery can be easily calculated. Using the titer of the spore inoculum as the baseline and factoring in the 10 L volume applied per carrier, the investigator can calculate percent recovery based on the volume applied versus the actual counts. For example, the lead laboratory in the validation study measured the titer of the spore inoculum to be 2.02 10^9 spores/mL. The mean log spore density per carrier for the lead laboratory was 6.85. Thus, approximately 94% of the total spores applied to the carrier were recovered across the 3 TSM fractions. The mean titer of spores/mL of inoculum across the laboratories was approximately 1.8 10^9.

HCl resistance test.—To qualify the spore preparation for use, each laboratory performed the HCl resistance test prior to performing the efficacy evaluations. Based on precollaborative results, a 1–2 and 3–5 LR in spore density per carrier was anticipated for the 2 and 5 min exposures, respectively. Per laboratory, the same batch of inoculated carriers was used for the HCl test and the efficacy component of the TSM. In this portion of the study, the mean log spore density per carrier for the control was 6.93 with $S_R = 0.28$, values very similar to the control counts for the efficacy tests. In each laboratory, the LR at 5 min was higher than the LR at 2 min. The means of LR were 1.8 and 3.9 for the 2 and 5 min exposure periods, respectively. *See* Table 3 for a summary of the HCl resistance data. The S_R values were somewhat elevated (near 1 for both the 2 and 5 min exposure). This reflects the increase in variability in LR data associated with intermediate efficacy test chemical treatments. Overall, the TSM HCl test accurately ranked the 2 and 5 min exposure periods at each laboratory. Based on our findings, the anticipated LR values for 2 and 5 min exposure in the TSM protocol should be adjusted to 0–3 and 2–6, respectively. Based on the data, the HCl test should be retained as a tool to qualify the spore preparation for use in efficacy testing.

Efficacy data.—The LR values generated by the TSM for each laboratory are presented in *Appendix C*. Based on the Cochran outlier test (15) as performed by an AOAC statistician, 4 significant outliers were discovered: (*1*) Laboratory 5 for the low sodium hypochlorite treatment; (*2*) Laboratory 7 for the high sodium hypochlorite treatment; (*3*) Laboratory 7 for the medium PA/HP treatment; and (*4*) Laboratory 7 for the high PA/HP treatment. In case (*1*), the variability among the replicates for Laboratory 5 was not unusual in the context of that observed for other treatments.

Table 5. For carriers receiving each test chemical treatment and for control carriers, the percentage of the TSM total count for all treated carriers that was attributable to each of Fractions A–C

Test chemical	Efficacy level	A, %	B, %	C, %
NaOCl	Low	6.1	93.0	0.8
NaOCl	Medium	41.0	55.5	3.5
NaOCl	High	37.8	42.2	20.0
PA/HP	Low	2.9	93.2	3.9
PA/HP	Medium	42.6	42.5	14.9
PA/HP	High	71.9	16.2	11.9
Glutaraldehyde	Low	18.8	80.0	1.3
Glutaraldehyde	Medium	33.5	64.7	1.8
Glutaraldehyde	High	60.8	31.4	7.8
Control	—	38.5	59.5	1.9

The outlier was due primarily to a large count at the first dilution of Fraction A on one carrier. The Study Director's investigation found no indication of anything unusual in the laboratory manipulations of that carrier. Cases (*2–4*) were similar in that the high variability among replications in Laboratory 7 was attributable to an unusually high count on a treated carrier. No errors or unusual events were recorded on the raw data sheets. The occurrences of the few variable counts noted are considered to be a component of the inherent variability of the method. If the data were edited prior to analysis by removing outlying data according to the Cochran test, the SDs would be lower. Because the data were not edited and all of the LRs were analyzed, the estimated repeatability and reproducibility SDs for TSM were conservatively higher; nevertheless, those SDs were acceptably low according to predetermined criteria. In addition, the control counts for the laboratories associated with the outliers were not unusual and were representative of the control count data set.

Based on the analysis of the TSM efficacy data, the TSM is a responsive, repeatable, and reproducible method. Across the 9 test chemical treatments, the mean LR ranged from 0.07 to 5.85. With the exception of one test chemical treatment, the TSM produced LR values that properly ordered the efficacy level for each chemical efficacy level combination (Figure 2). The TSM consistently showed an increase in LR from the low to high levels and the amount of increase was also repeatable. The exception was the PA/HP, where the medium efficacy level was as effective as the high efficacy level treatment; however, the pattern was consistent across laboratories. The TSM tracked very closely to Method **966.04**, including measuring the similarity in the performance of the medium and high PA/HP treatments.

A summary of the efficacy data is provided in Table 4. The mean LR values ranged from 0.07 to 5.85. The S_r and S_R estimates were within acceptable ranges; the S_r ranged from 0.17 to 0.72 and the S_R ranged from 0.34 to 1.43. For test chemical treatments that produced mean LR between 1.0 and 4.0, the among-laboratory variation was a major contributor to the total variation. Overall, the S_r and S_R estimates associated with the efficacy data were within the ranges published for other quantitative methods and meet the performance characteristics necessary for validation.

Contribution of Fractions A–C

In an effort to optimize and possibly shorten the TSM, an analysis of the contribution of each fraction to the total number of spores recovered was performed. The greatest interest was the contribution of Fraction C to the total viable count. Historically, Fraction C has contributed the fewest number of viable spores to the total. For the control carriers, Fraction C had a negligible impact on the total number of spores recovered. The percentage of the total treated and control carrier counts attributable to each fraction are shown in Table 5. Overall, the contribution of Fraction C increases with product efficacy and was shown to be as high as 20% for the high sodium hypochlorite treatment. The contribution of Fraction C to the low and medium levels was less important and ranged from 0.8 to 14.9% of the total. The medium and high PA/HP exhibited similar amounts of Fraction C contribution. Based on these findings, the Fraction C protocol of the TSM should be retained.

*Comparative Assessment of the Three Step Method and AOAC Method **966.04***

Method **966.04** was included in the collaborative study to provide baseline data to assess the performance of the TSM. Because Method **966.04** provides a qualitative response (positive/negative) and not a direct quantitative response, only approximate or visual comparisons were possible. The responsiveness of both methods (i.e., the ability to discriminate between treatments of different efficacy) is displayed in Figures 1 and 2. The mean number of positive carriers for Method **966.04** decreased with increasing efficacy level for each test chemical (Figure 1) and the mean LR for TSM increased with increasing efficacy levels (Figure 2). The TSM was unable to detect a difference between the medium and high efficacy levels of PA/HP, an unanticipated result based on prevalidation data. Because Method **966.04** produced a similar result, it appears that the TSM was correct in showing that the medium PA/HP treatment was about as efficacious as the high treatment.

To aid in the assessment, qualitative data for Method **966.04** were converted into LR estimates using the P/N formula, and the mean LR values were plotted against efficacy levels (Figure 2). The P/N conversion is not an official component of Method **966.04**. In this application, the P/N formula was used as a means to evaluate the 2 methods. Because LR values for Method **966.04** were truncated at around 5, the P/N formula approach for Method **966.04** was less effective in differentiating between low and medium efficacy treatments. The slopes of the lines connecting medium to high efficacy for each test chemical may be used in the evaluation of responsiveness for the TSM and Method **966.04**. Slopes for Method **966.04** and TSM are visually similar, indicating that the responsiveness of the TSM was comparable to Method **966.04** for discriminating between medium and high efficacy treatments (Figure 2). Clearly, TSM was more effective at discriminating between low and medium efficacy levels. Overall, the P/N formula LR for Method **966.04** was higher than the corresponding LR for the TSM. The laboratory test procedures and the formulas for calculating LR are different for the 2 methods and one would not expect them to produce the same LR.

In addition, the P/N formula provided a way to calculate S_R for Method **966.04** data in the same units as the S_R for TSM. For the 4 treatments that provided Method **966.04** responses

suitable for calculating S_R, the range of Method **966.04** S_R values was 0.48 to 0.78. The corresponding TSM S_R values were somewhat higher, ranging from 0.65 to 1.23. Method **966.04** was modified for this collaborative study to use only 30 disinfected carriers instead of 60. Because the variation of the number of positive carriers was somewhat narrow, the S_R observed in this study may underestimate the S_R for the 60 carrier Method **966.04**.

Conclusions

In order to determine the reliability of the TSM, a multilaboratory validation study was conducted. As measures of method performance, low S_r and S_R are desirable; however, acceptable levels have not been fully defined or published for antimicrobial product test methods by AOAC INTERNATIONAL. In the precollaborative study involving 2 quantitative methods (6), the S_r ranged from 0.25 to 0.66 and the S_R ranged from 0.26 to 1.12. A similar outcome was observed in the TSM validation study. In a review of published quantitative efficacy data by Tilt and Hamilton (11), the ranges of S_r and S_R were 0.25–1.21 and 0.31–1.54, respectively. The literature review included the outcome of a 15 laboratory collaborative study on a version of the Standard Quantitative Carrier Test Method (QCT-1). The data analysis (16) showed that the majority of variability exhibited with QCT-1 was between laboratories and not within laboratories. For QCT-1, the S_r values were 0.19, 0.84, and 0.25 for weak, intermediate, and strong sporicides, respectively. The S_R values were 0.58, 1.31, and 0.50 for weak, intermediate, and strong sporicides, respectively. These values demonstrate that marginal products generate more variable results than products with stronger formulations. Overall, Tilt and Hamilton determined that the laboratory-to-laboratory variability was the largest contributor to the overall S_R, and they emphasized the need for multilaboratory studies. Similarly, increased variability was also associated with the TSM efficacy data for the test chemicals with medium (intermediate) efficacy. Based on the variability reported by Tilt and Hamilton, the TSM exhibited low and acceptable S_r and S_R values.

The TSM, evaluated according to the conditions of this collaborative study, successfully met the statistical parameters described in this report. Satisfactory validation parameters for control and treated carriers such as repeatability and reproducibility were obtained for controls and LR values associated with the test chemical treatments. Based on the results, the TSM was responsive to the change in efficacy of the test chemicals and acceptably repeatable. Both TSM and Method **966.04** found that the medium and high levels of PA/HP produced about the same high LR.

The target range of the TSM control counts was successfully obtained in the majority of the laboratories. Despite the fact that a minority of the mean control counts measured in the study (lowest measured was 6.3 logs spores/carrier) was less than the proposed target (minimum of 6.7 logs of spores/carrier minimum), we believe that this difference is not of practical importance. A minor editorial change to the protocol instructing the user to dilute the spore inoculum less, thus increasing the titer and the carrier counts, is recommended in the final version. Resistance of spores to 2.5 M HCl, as measured with the TSM, showed consistent differences in LR between the 2 and 5 min exposures and will be a useful tool to qualify the spores prior to use in the efficacy test. Also, based on the prevalidation surrogate studies, we recommend broadening the TSM applicability statement in the final published version to specifically include use for testing strains of *B. anthracis*.

The use of the TSM as a reference method for the validation of other quantitative tests for sporicides is encouraged. The EPA, in conjunction with AOAC INTERNATIONAL, incorporated video clips into the TSM protocol. The video clips demonstrate selected procedures for conducting the TSM in an effort to further standardize the procedure and reduce user error. In addition, it is recommended that AOAC INTERNATIONAL make available the electronic spreadsheets used in the validation to facilitate data summary and LR calculation process. In the future, it is anticipated that the TSM protocol will be expanded to include additional coupon materials to represent porous surfaces relevant to buildings and environmental surfaces (e.g., wood, ceiling tile, concrete). Also, it is anticipated that the use of the TSM to evaluate the performance of gaseous sporicidal formulations will be pursued. The validation of porous carrier materials and gaseous product formulation will require additional collaborative studies.

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Appendix A. Number of positive carriers out of 30 tested and the associated LR for Method 966.04, presented for each level of each test chemical in each laboratory

Lab	Test chemical	Level	No. positive carriers	Control mean log density	LR (P/N formula)
1	NaOCl	Low	30	5.70155	5.08590
1	NaOCl	Medium	23	5.70155	5.54954
1	NaOCl	High	0	5.70155	7.49041
1	PA/HP	Low	30	5.70155	5.08590
1	PA/HP	Medium	0	5.70155	7.49041
1	PA/HP	High	0	5.70155	7.49041
1	Glut	Low	29	5.70155	5.22032
1	Glut	Medium	13	5.70155	5.94431
1	Glut	High	8	5.70155	6.19576
2	NaOCl	Low	30	5.93819	5.32254
2	NaOCl	Medium	30	5.93819	5.32254
2	NaOCl	High	0	5.93819	7.72705
2	PA/HP	Low	30	5.93819	5.32254
2	PA/HP	Medium	0	5.93819	7.72705
2	PA/HP	High	0	5.93819	7.72705
2	Glut	Low	30	5.93819	5.32254
2	Glut	Medium	3	5.93819	6.85972
2	Glut	High	1	5.93819	7.24273
3	NaOCl	Low	30	4.92635	4.31070
3	NaOCl	Medium	3	4.92635	5.84789
3	NaOCl	High	0	4.92635	6.71522
3	PA/HP	Low	30	4.92635	4.31070
3	PA/HP	Medium	3	4.92635	5.84789
3	PA/HP	High	0	4.92635	6.71522
3	Glut	Low	30	4.92635	4.31070
3	Glut	Medium	4	4.92635	5.73089
3	Glut	High	3	4.92635	5.84789
4	NaOCl	Low	30	5.02275	4.40711
4	NaOCl	Medium	30	5.02275	4.40711
4	NaOCl	High	0	5.02275	6.81162
4	PA/HP	Low	30	5.02275	4.40711
4	PA/HP	Medium	1	5.02275	6.32730
4	PA/HP	High	0	5.02275	6.81162
4	Glut	Low	30	5.02275	4.40711
4	Glut	Medium	30	5.02275	4.40711
4	Glut	High	3	5.02275	5.94429
5	NaOCl	Low	30	5.82350	5.20785
5	NaOCl	Medium	17	5.82350	5.90374
5	NaOCl	High	0	5.82350	7.61236
5	PA/HP	Low	30	5.82350	5.20785
5	PA/HP	Medium	4	5.82350	6.62804
5	PA/HP	High	0	5.82350	7.61236
5	Glut	Low	30	5.82350	5.20785
5	Glut	Medium	30	5.82350	5.20785
5	Glut	High	0	5.82350	7.61236
6	NaOCl	Low	30	5.47934	4.86369
6	NaOCl	Medium	25	5.47934	5.24149
6	NaOCl	High	0	5.47934	7.26821
6	PA/HP	Low	30	5.47934	4.86369
6	PA/HP	Medium	4	5.47934	6.28388
6	PA/HP	High	3	5.47934	6.40088
6	Glut	Low	30	5.47934	4.86369
6	Glut	Medium	30	5.47934	4.86369
6	Glut	High	7	5.47934	6.03688
7	NaOCl	Low	30	5.82875	5.21310
7	NaOCl	Medium	30	5.82875	5.21310
7	NaOCl	High	1	5.82875	7.13329
7	PA/HP	Low	30	5.82875	5.21310
7	PA/HP	Medium	14	5.82875	6.02898
7	PA/HP	High	2	5.82875	6.90404
7	Glut	Low	30	5.82875	5.21310
7	Glut	Medium	30	5.82875	5.21310
7	Glut	High	23	5.82875	5.67674
8	NaOCl	Low	30	5.40983	4.79418
8	NaOCl	Medium	28	5.40983	5.00883
8	NaOCl	High	1	5.40983	6.71438
8	PA/HP	Low	30	5.40983	4.79418
8	PA/HP	Medium	0	5.40983	7.19870
8	PA/HP	High	0	5.40983	7.1987
8	Glut	Low	30	5.40983	4.79418
8	Glut	Medium	30	5.40983	4.79418
8	Glut	High	14	5.40983	5.61006

Appendix B. TSM control counts (log_{10} density) for each carrier in each laboratory/day

Lab	Day	Log density
1	1	6.916262
1	1	6.848245
1	1	6.993236
1	2	6.71265
1	2	6.754626
1	2	6.793854
1	3	6.738492
1	3	6.82056
1	3	6.938156
1	4	6.662758
1	4	6.739572
1	4	6.740865
1	5	7.125215
1	5	6.842098
1	5	6.871626
1	6	6.846281
1	6	7.058219
1	6	7.026422
1	7	6.853256
1	7	6.845718
1	7	6.771253
1	8	6.753513
1	8	7.005102
1	8	6.700389
1	9	6.91564
1	9	6.745572
1	9	6.897577
2	1	6.893257
2	1	7.003225
2	1	6.929744
2	2	6.792837
2	2	6.92667
2	2	7.129866
2	3	7.026199
2	3	7.0044
2	3	7.030673
2	4	6.953935
2	4	6.976433
2	4	6.928349
2	5	6.952572
2	5	6.867307
2	5	6.924796
2	6	7.073885
2	6	7.028497
2	6	7.08568
2	7	6.857881
2	7	7.060217
2	7	6.805254
2	8	6.660175
2	8	6.929001
2	8	6.958434
2	9	6.916023
2	9	6.900069
2	9	6.937974
3	1	7.531943
3	1	7.209442
3	1	7.235666
3	2	7.1801
3	2	7.58227
3	2	6.982888
3	3	7.467756
3	3	7.403417
3	3	7.347773
3	4	7.327582
3	4	7.613525
3	4	7.274704
3	5	6.986038
3	5	7.076773
3	5	7.168605
3	6	7.458528
3	6	7.351304
3	6	6.964859
3	7	6.885772
3	7	6.951072
3	7	7.115096
3	8	7.235023
3	8	7.350054
3	8	7.292115
3	9	7.404445
3	9	7.20518
3	9	7.19459
4	1	6.879409
4	1	6.68362
4	1	6.700625
4	2	6.711807
4	2	6.447017
4	2	6.738925
4	3	6.539076
4	3	6.623061
4	3	6.437895
4	4	6.840163

Appendix B. (*continued*)

Lab	Day	Log density
4	4	6.745997
4	4	6.759874
4	5	6.517915
4	5	6.389971
4	5	6.603931
4	6	6.073885
4	6	6.321655
4	6	6.303392
4	7	6.112727
4	7	6.220346
4	7	6.450389
4	8	6.699917
4	8	6.376909
4	8	6.261155
4	9	6.391737
4	9	6.738132
4	9	6.649689
5	1	6.771654
5	1	7.203478
5	1	6.982436
5	2	6.97022
5	2	7.093804
5	2	6.778414
5	3	6.793727
5	3	6.716079
5	3	6.888282
5	4	6.716534
5	4	6.915927
5	4	6.76438
5	5	7.434438
5	5	7.402418
5	5	7.096594
5	6	7.459351
5	6	7.009567
5	6	6.791754
5	7	6.804201
5	7	6.715548
5	7	6.76336
5	8	7.485528
5	8	7.004204
5	8	7.704166
5	9	7.124386
5	9	6.80618
5	9	6.80028
6	1	6.738348
6	1	6.726357

Appendix B. (*continued*)

Lab	Day	Log density
6	1	6.668301
6	2	6.77894
6	2	6.766886
6	2	6.905158
6	3	6.675362
6	3	6.840448
6	3	6.722484
6	4	6.788361
6	4	6.710117
6	4	6.497181
6	5	6.772988
6	5	6.65723
6	5	6.817505
6	6	6.735018
6	6	6.652598
6	6	6.643453
6	7	6.617382
6	7	6.50478
6	7	6.640752
6	8	6.536558
6	8	6.553
6	8	6.580199
6	9	6.607162
6	9	6.748399
6	9	6.581546
7	1	6.955731
7	1	6.744364
7	1	6.837301
7	2	7.128311
7	2	7.075779
7	2	6.933257
7	3	6.779203
7	3	6.993356
7	3	6.357588
7	4	7.173557
7	4	7.149331
7	4	7.233342
7	5	6.817625
7	5	6.50589
7	5	6.727245
7	6	6.573926
7	6	6.898376
7	6	6.69968
7	7	7.034665
7	7	6.918316
7	7	6.753861

Appendix B. (*continued*)

Lab	Day	Log density
7	8	7.315665
7	8	7.271884
7	8	7.361814
7	9	6.985304
7	9	7.077797
7	9	7.520507
8	1	6.700704
8	1	6.72353
8	1	6.752188
8	2	6.668555
8	2	6.668894
8	2	6.883868
8	3	6.722634
8	3	6.667283
8	3	6.59389
8	4	6.528917
8	4	6.684682
8	4	6.677939
8	5	6.641113
8	5	6.745784
8	5	6.701254
8	6	6.5302
8	6	6.561643
8	6	6.955557
8	7	6.802401
8	7	6.855739
8	7	6.586485
8	8	6.634294
8	8	6.726727
8	8	6.556851
8	9	6.593588
8	9	6.692206
8	9	6.772522

Appendix C. TSM LR value for each of the 3 efficacy levels of a test chemical, presented for each replication of each test chemical in each laboratory

Lab	Test chemical	Replicate	Efficacy		
			Low	Medium	High
1	NaOCl	1	0.16821	3.69694	6.13343
1	NaOCl	2	0.01941	3.65784	6.01543
1	NaOCl	3	0.12837	4.14487	6.15396
2	NaOCl	1	0.07750	2.43009	6.32145
2	NaOCl	2	0.08409	2.90087	6.36372
2	NaOCl	3	0.01632	2.65767	6.11871
3	NaOCl	1	0.32829	4.12731	6.52637
3	NaOCl	2	0.72564	3.57767	6.15789
3	NaOCl	3	0.47932	4.42324	5.36726
4	NaOCl	1	0.78844	5.45949	5.93361
4	NaOCl	2	1.60978	5.02066	6.08304
4	NaOCl	3	1.41326	5.80767	5.89422
5	NaOCl	1	–0.40546[a]	3.99223	4.55017
5	NaOCl	2	1.11109[a]	4.53039	5.17609
5	NaOCl	3	1.43779[a]	4.51527	4.97573
6	NaOCl	1	0.71812	3.40196	5.35407
6	NaOCl	2	0.48227	3.77996	5.66522
6	NaOCl	3	0.70884	5.13558	5.14399
7	NaOCl	1	0.40782	2.39501	4.07137[a]
7	NaOCl	2	0.80087	2.99518	4.75977[a]
7	NaOCl	3	0.52531	3.03630	6.49557[a]
8	NaOCl	1	0.42582	3.86668	5.96230
8	NaOCl	2	0.44831	4.05672	5.93154
8	NaOCl	3	0.84419	4.43604	5.98714
1	PA/HP	1	0.12777	6.11993	6.11993
1	PA/HP	2	0.43715	6.27800	6.27800
1	PA/HP	3	–0.05059	6.12444	6.12444
2	PA/HP	1	0.05140	6.24311	6.24311
2	PA/HP	2	0.08776	6.25394	6.25394
2	PA/HP	3	–0.24206	6.15023	6.15023
3	PA/HP	1	3.71697	6.03068	6.00163
3	PA/HP	2	1.62770	6.11878	5.57318
3	PA/HP	3	1.22019	5.73426	4.86047
4	PA/HP	1	0.33312	5.95524	6.05558
4	PA/HP	2	0.58065	5.53401	5.53401
4	PA/HP	3	0.83785	5.74702	5.74702
5	PA/HP	1	2.65648	6.24851	6.24851
5	PA/HP	2	4.68588	6.61218	6.31115
5	PA/HP	3	3.05067	5.86138	5.66069
6	PA/HP	1	2.92549	5.40997	5.01794
6	PA/HP	2	2.36746	6.05027	6.05027
6	PA/HP	3	2.77818	5.55659	5.65693
7	PA/HP	1	1.25698	4.07776[a]	6.34681[a]
7	PA/HP	2	2.85193	3.91810[a]	3.53949[a]
7	PA/HP	3	1.52489	6.61748[a]	6.61748[a]
8	PA/HP	1	0.29764	5.74044	6.04147
8	PA/HP	2	0.48832	5.89674	5.99708
8	PA/HP	3	0.34420	6.04924	5.94890
1	Glut	1	–0.02873	4.55688	6.05474
1	Glut	2	0.20799	4.80843	6.14700
1	Glut	3	–0.22884	4.35228	6.12070
2	Glut	1	–0.05047	5.52754	6.25082
2	Glut	2	–0.00839	5.27848	6.11558
2	Glut	3	–0.16111	4.91897	6.20881
3	Glut	1	0.24387	4.02034	4.91454
3	Glut	2	–0.16492	2.91645	5.80321
3	Glut	3	0.00309	3.91833	4.82533
4	Glut	1	–0.35293	1.85299	4.77452
4	Glut	2	–0.39931	2.72537	5.80497
4	Glut	3	–0.59743	2.50147	5.30280
5	Glut	1	0.93906	3.57554	4.80347
5	Glut	2	0.55642	3.18772	4.46816
5	Glut	3	0.44803	4.30084	4.72485
6	Glut	1	0.31517	3.78574	5.81700
6	Glut	2	0.34009	2.50447	5.97805
6	Glut	3	0.20512	5.13666	5.88867
7	Glut	1	–0.00853	1.65261	4.10979
7	Glut	2	0.08221	1.70935	3.79594
7	Glut	3	0.41119	3.61482	5.82127
8	Glut	1	–0.00600	4.58671	5.66678
8	Glut	2	0.03544	4.85763	5.98350
8	Glut	3	–0.02767	5.26226	5.94032

[a] Determined to be a Cochran test outlier; however, no outliers were excluded from the data analysis and standard deviation estimates.