UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
PREVENTION,
PESTICIDES
AND TOXIC
SUBSTANCES

May 15, 2009

MEMORANDUM

Subject: Protocol Review for EPA File Symbol 81073-E, Peridox with the Electrostatic Decontamination System
DP Barcode: 361712

From: Tajah L. Blackburn, Ph.D., Microbiologist
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Thru: Michele Wingfield, Chief
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To: Marshall Swindell PM 33/ Karen Leavy
Regulatory Management Branch I
Antimicrobials Division (7510P)

Applicant: Clean Earth Technologies, LLC
13378 Lakefront Drive
St. Louis, MO 63045

Formulation from the Label:

Active Ingredient(s)	% by wt.
Hydrogen Peroxide	24.0%
Peroxyacetic Acid	1.2%
Other Ingredients	74.8%
Total	100.0 %

I BACKGROUND

This data package is submitted in response to several EPA correspondences as well as conference calls between Clean Earth Technologies (CET) and EPA regarding the Surface Sterilization Test (SST) protocol. According to the registrant's representative's letter, "your [the EPA] October 6, 2008, letter requests changes to the product label as well as the instruction manual. CET has made the EPA requested changes as well as a few additional changes that are highlighted. These additional changes were made as a result of subsequent discussions with the Agency as well as minor changes to clarify use directions." Additionally, on October 8, 2008, "a conference call was held between EPA and CET to discuss a September 30, 2008, memorandum and technical review prepared by Dr. Stephen Tomasino and Michele Wingfield. During the call, CET discussed the review and the six recommendations made by Dr. Tomasino for additional information on the SST method as well as additional data. Based on the discussion, supplemental information as requested is provided in Appendix A of this letter. Additional data were generated by ATS Laboratories and the data reports were provided in Volumes 2 and 3 of the current submission." Furthermore, the Agency requested information comprises the following:

Item 1	Specific revisions of the Master Label and Instruction Manual
Item 2	Protocols for spore production
Item 3	Details on the SST neutralizer controls and the associated results
Item 4	Details of sporulation media and spore quality control indicators used in the comparative study reported on page 14 of the previously submitted Efficacy & Method Validation Report
Item 5	Additional efficacy data per GLP standards with one Bacillus species and three batch lots, one of which is at least 60 days old
Item 6	Empirical evidence to support the use of a hand-held sprayer in the SST instead of the specialized wand.

Note: Per the letter submitted in the current data package, "the reason why two volumes are submitted with this response is related to the results of one lot on the glass carriers. While normally the lab would have repeated the test for the one lot under the same study, it was unclear whether the repeat test would have been completed by the PRIA deadline of January 23, 2009. Therefore, CET requested that ATS Labs issue two separate reports so that the main study report would have been submitted to meet the deadline. However, ATS Labs worked diligently and was able to repeat the testing for the one lot quickly and issue a second report. Therefore, the two reports represent all the testing and data generated to respond to the recommendations."

Note: Per the letter submitted in the current data package, "evidence to support the use of the a hand-held sprayer in the SST was previously submitted to the Agency, and notification of the Agency concurrence on the use of the hand-held sprayer was given on November 19, 2008."

II USE DIRECTIONS (from Instructions Manual)

The EDS process plus Peridox has claims as a sporicidal decontaminate to treat dry, pre-cleaned, hard, non-porous surfaces contaminated with *Bacillus anthracis* spores including buildings, structures, vehicles, aircraft, PPE, and articles/items. Directions on the proposed label provided the following instructions for the preparation and use of the product as a sterilant:

> The EDS process requires 2 steps: Step 1 is wetting of the surface to be decontaminated with a 4% Use Dilution solution of Peridox by spray application at a rate of 1-2 oz/yd^2 (0.1-0.25 oz/ft^2). A use dilution solution comprising 52 oz of Peridox concentrate and 2 gallons of water (amounting to a total volume of 2.4 gallons, which is the reservoir capacity of the EDS Delivery Unit) will treat approximately 1385-2770 ft^2. After a contact time of at least three (3) minutes, proceed to Step 2, which is the illumination of the wetted surface by the EDS Light Wand at a distance of less than two (2) feet from the surface and with a motion across the surface at a rate of not more than one (1) foot per second. The surface after applying the 4% use dilution solution must remain wet for the entire time prior to illumination. Reapply 4% use dilution by spraying as necessary to maintain the wet surface. Illuminate the wet surface within ten (10) minutes of the spray application.

III AGENCY STANDARDS FOR PROPOSED CLAIMS

<u>Sterilizers</u>

The AOAC Sporicidal Test is required for substantiating sterilizing claims. The following information applies to all products represented as sporicidal or sterilizing agents. Sixty carriers, representing each of 2 types of surfaces (porcelain penicylinders and silk suture loops), must be tested against spores of both *Bacillus subtilis* (ATCC 19659) and *Clostridium sporogenes* (ATCC 3584) on 3 product samples representing 3 different product lots, one of which is at least 60 days old (240 carriers per sample; a total of 720 carriers). Any sterilizing agent (liquid, vapor, or gas) that is recommended for use in a specific device must be tested by the AOAC Sporicidal Test in that specific device and according to the directions for use. Killing on all of the 720 carriers is required; no failures are permitted. Data to support sterilizing claims must be confirmed by tests conducted by a second, independent laboratory of the applicant's choice (other than the laboratory that developed the original data). The following minimal confirmatory data must be developed on one sample of the product: Thirty carriers with each of 2 types of surfaces (silk suture loops and porcelain penicylinders) against spores of both *Bacillus subtilis* and *Clostridium sporogenes* (a total of 120 carriers) by the AOAC Sporicidal Test.

IV SYNOPSIS OF SUBMITTED STUDIES

1. MRID No. 476499-01, "Surface Sterilization Test Method" Against *Bacillus subtilis* (ATCC 19659) by Amy Jeske. Study completion date—January 19, 2009. Project Number—A07187.

This study was conducted against *Bacillus subtilis* (ATCC 19659) using ATS Lab Protocol No. CTL0 t t21608.CUST (protocol attached). Three lots (Lot Nos. CET090308SI84, CET050808SI70, AND CET032708SI65) of the product, Peridox, were tested. No lot was designated the aged lot. The test substance was prepared by adding t part Peridox to 5 parts 200 ppm AOAC Synthetic Hard Water. Product was tested in the presence organic soil (Tryptone, BSA, and bovine mucin soil). Two different carrier types were used in the test: glass Petri dishes and aluminum weighing dishes. Prior to sterilization, a permanent marker was used to draw a 3.5 cm diameter circle on the bottom interior surface of the aluminum dishes and on the bottom exterior of the glass Petri dishes. Ten glass dishes and ten aluminum dishes, for each lot tested, were inoculated with 100 µl of the prepared inoculum. The inoculum was applied to the inside center of the dishes using a calibrated pipettor. A sterile 10 µl loop was used to spread the inoculum over the entire 3.5 cm circle. The contaminated dishes, with the lids open, were placed in desiccators containing active desiccant. Carriers were dried under vacuum at room temperature (t8°C) for one hour. At the end of the drying period, the vacuum was turned off and the dishes were removed from the desiccators and held at room temperature until used in testing. Each spray bottle and spray nozzle was sterilized inside and out by submerging the disassembled sprayer in 1% solution of Peridox for one hour. At staggered intervals, t0 dried bacterial spore films on both the glass Petri dishes and the aluminum dishes were sprayed with the test substance. The Petri dishes were each sprayed with the use dilution of the test substance using a minimum 9 forceful full-stroke pump spray actuations with the spray bottle at a distance of 2-2.5" from the carrier surface. Aluminum carriers were each sprayed with the use dilution of the test substance using 16 forceful half-stroke pump spray actuations with the spray bottle at a distance of 2-2.5" from the carrier's surface. On both carrier types, between each spray, the dish was rotated approximately ¼ turn. After spraying, each dish was held at room temperature (t9°C and t2% relative humidity) for 3 minutes before being exposed to the computer controlled pulsed UV light system with a fluence of 28-33 mjoules/cm^2. The number of flashes and the fluence reading produced by the computer controlled pulsed UV light was recorded. Following the exposure period, 1.20 ml of neutralizer was applied to each treated glass Petri dish and 2.00 ml of neutralizer was applied to each treated aluminum dish. An aliquot (3.0 ml was added to the glass Petri dishes and 7.2 ml was added to the aluminum dishes) of wash solution was added to each dish. The entire volume of liquid in the dish was collected and transferred to a sterile tube. An additional 5.0 ml of wash solution 1 was added to the glass Petri dishes only, to rinse any remaining spores from the surface. This was collected and added to the corresponding 10^0 tube. Ten-fold serial dilutions of the neutralized suspension were made in sterile 0.85% saline through the $t0^{-3}$ dilution. Individual sterile filter units with 0.45 µm porosity were wetted with t0.0 ml sterile 0.85% saline. The entire volume remaining in the 10^0 tube following the serial dilutions (~9 ml) was pipetted on to the surface of a filter membrane. In addition, 9.0 ml of each of the dilutions ($t0^{-1}$ to 10^{-3}) was pipetted onto the surface of separate filter membranes and filtered. The collection/dilution tubes and the filter membranes were sequentially washed by adding at

least 50 ml of sterile saline to the tubes and then transferring this volume to the appropriate filter unit/pump apparatus and evacuating. Each filter membrane was aseptically removed from the filter unit and placed on the surface of a TSA plate with 5% sheep blood agar. Subcultures were incubated at 35-37° for approximately 44 hours. Following incubation, plates were enumerated. All plates were returned to the incubator and incubated at 35-37°C for an additional 3 days and enumerated a second time. Any plates with colonies that had grown together or which were otherwise not countable were discarded. Upon completion of the entire incubation (5 days), representative culture plates demonstrating growth were appropriately examined for confirmation of the test organism. Controls included those for purity, sterility, neutralization confirmation, and initial suspension/carrier population control.

Protocol deviations/amendments were reviewed.

2. MRID No. 476499-02, "Surface Sterilization Test Method" Against *Bacillus subtilis* (ATCC 19659) by Amy Jeske. Study **completion date—January 22, 2009. Project Number—A07226.**

This study was conducted against *Bacillus subtilis* (ATCC 19659) using ATS Lab Protocol No. CTL01011209.CUST (protocol attached). One lot (Lot No. CET032708SI65) of the product, Peridox, was tested. The test substance was prepared by adding 1 part Peridox to 5 parts 200 ppm AOAC Synthetic Hard Water. Only glass Petri dishes were used as the carriers. Prior to sterilization, a permanent marker was used to draw a 3.5 cm diameter circle on the bottom exterior of the glass Petri dishes. Ten glass dishes, for one lot tested, were inoculated with 100 µl of the prepared inoculum. The inoculum was applied to the inside center of the dishes using a calibrated pipettor. A sterile 10 µl loop was used to spread the inoculum over the entire 3.5 cm circle. The contaminated dishes, with the lids open, were placed in desiccators containing active desiccant. Carriers were dried under vacuum at room temperature (18°C) for one hour. At the end of the drying period, the vacuum was turned off and the dishes were removed from the desiccators and held at room temperature until used in testing. Each spray bottle and spray nozzle was sterilized inside and out by submerging the disassembled sprayer in 1% solution of Peridox for one hour. At staggered intervals, 10 dried bacterial spore films on both the glass Petri dishes and the aluminum dishes were sprayed with the test substance. The Petri dishes were each sprayed with the use dilution of the test substance using a minimum 9 forceful full-stroke pump spray actuations with the spray bottle at a distance of 2-2.5" from the carrier surface. Carriers, between each spray, were rotated approximately ¼ turn. After spraying, each dish was held at room temperature (19°C and 12% relative humidity) for 3 minutes before being exposed to the computer controlled pulsed UV light system with a fluence of 28-33 mjoules/cm^2. The number of flashes and the fluence reading produced by the computer controlled pulsed UV light was recorded. Following the exposure period, 1.20 ml of neutralizer was applied to each treated glass Petri dish. An aliquot (3.0 ml was added to the glass Petri dishes) of wash solution was added to each dish. The entire volume of liquid in the dish was collected and transferred to a sterile tube. An additional 5.0 ml of wash solution 1 was added to the glass Petri dishes only, to rinse any remaining spores from the surface. This was collected and added to the corresponding 10^0 tube. Ten-fold serial dilutions of the neutralized suspension were made in sterile 0.85% saline through

the 10^{-3} dilution. Individual sterile filter units with 0.45 µm porosity were wetted with 10.0 ml sterile 0.85% saline. The entire volume remaining in the 10^{0} tube following the serial dilutions (~9 ml) was pipetted on to the surface of a filter membrane. In addition, 9.0 ml of each of the dilutions (10^{-1} to 10^{-3}) was pipetted onto the surface of separate filter membranes and filtered. The collection/dilution tubes and the filter membranes were sequentially washed by adding at least 50 ml of sterile saline to the tubes and then transferring this volume to the appropriate filter unit/pump apparatus and evacuating. Each filter membrane was aseptically removed from the filter unit and placed on the surface of a TSA plate with 5% sheep blood agar. Subcultures were incubated at 35-37° for approximately 44 hours. Following incubation, plates were enumerated. All plates were returned to the incubator and incubated at 35-37°C for an additional 3 days and enumerated a second time. Any plates with colonies that had grown together or which were otherwise not countable were discarded. Upon completion of the entire incubation (5 days), representative culture plates demonstrating growth were appropriately examined for confirmation of the test organism. Controls included those for purity, sterility, neutralization confirmation, and initial suspension/carrier population control.

Note: In order to accommodate the Sponsor requested scheduling and the weekend work time, the incubation and enumeration of the subculture is being changed to the following: Subculture will be incubated at 35-37°C for 2 days. Following the 2-day incubation, the test and control plates will be enumerated. All plates will then be incubated under appropriate conditions for an additional 3 days and enumerated a second time. Any plates with colonies that have grown together or are otherwise not countable will be discarded and the counts recorded from the 2 day incubation will be used for calculations. Upon completion of the entire incubation (5 days), representative culture plates demonstrating growth will be appropriately examined for confirmation of the test organism.

V Appendix A Supplemental Information in Response to the Technical Review by Dr. S. Tomasino.

Item 2: Protocols for spore production

Two types of spores have been used for studies with the SST to generate data previously submitted to the Agency. These two types are *Bacillus atrophaeus* and *Bacillus subtilis*. The Bs spores have also been used for recent SST studies at ATS Labs, Inc., which provided data that are enclosed in this submission. The relevant spore preparation methods and media were included.

Bg Spore Preparation Method—BCO

Bg spores were grown in fermentor batches in modified G (modG) medium (yeast extract, 2.0 g/L; $(NH_4)_2SO_4$, 2.0 g/L; $CaCl_2 \cdot 2H_2O$, 25 mg/L; $CuSO_4 \cdot 5H_2O$, 5.0 mg/L; $FeSO_4 \cdot 7H_2O$, 0.5 mg/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $MnSO_4 \cdot 4H_2O$, 50 mg/L; $ZnSO_4 \cdot 7H_2O$, 5.0 mg/L) at 30°C.

BCO routinely generates spore batches using 15 L fermentors. The culture is scaled-up in Tryptic Soy Broth (TSB) and then inoculated into modG broth (inoculated at a ratio of 1:100). The culture generally reaches stationary phase within the 8-12 hours at a concentration of approximately 5E + 8 CFU/ml. BCO then allows it to sporulate to >95% (based on microscopic observations). BCO observes some endospores within the first

24 hours and by 48 hours the culture is ready to be harvested. To do a shake-flask growth curve, BCO recommends growing a seed culture in TSB and then inoculating 1:100 in modG. Bg sporulates best at 30°C at 200 rpm. BCO generally grows cultures in flasks at ratio of 1 part medium volume to 5 parts flask volume (50 ml in 250 ml flask, for example).

Modified G (modG) Medium

Ingredient	Amount for 1 Liter
Yeast Extract	2.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCl_2 \cdot 2H_2O$	0.025 g
$CuSO_4 \cdot 5H_2O$	5.0 mg
$FeSO_4 \cdot 7H_2O$	0.5 mg
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot 4H_2O$	0.05 g
$ZnSO_4 \cdot 7H_2O$	5.0 mg
K_2HPO_4	0.5 g

Directions:

1. Dissolve yeast extract in 200 ml dH2O, and autoclave 15 minutes
2. Dissolve all ingredients, in the order listed, into 800 ml dH2O. [Allow chemical to dissolve completely before adding the next chemical.]
3. Aseptically combine both sterilized solutions to complete the medium.

Bs Spore Production and Suspension Preparation—Presque Isle Cultures

The following information was provided by Dr. Richard Gammon of Presque Isle Cultures. This information comprises a general description of the spore production for Bacillus subtilis (ATCC 19659) for lot 101904BS spore suspension in aqueous ethanol.

Reference:

Bacillus subtilis (ATCC 19659) Spore Production

C.H. Shaffer, Jr. PRD 66. [IFO 13722] Sporicidal Test (AOAC Methods 966.04, 1990). Testing of antimicrobial hand washing formulations (ASTM E1327-90). (Medium 3 30°C) Shipped Freeze-dried.

Per Dr. Gammon:

B. subtilis is cultured on fortified nutrient agar with $MnSO_4$, incubated at 37°C for 5 days at which time 95% sporulation is achieved. Spores are harvested, centrifuged, and washed with cold sterilized deionized water. After 3 washes, the spores are suspended in 40% aqueous ethanol and tittered using a standard dilution method and plating of dilutions in TSA.

Preparation of a **Bacterial Spore** Suspension

Materials:

1. Stock culture of lyophilized cultures of spore producing organism
2. Tubes/flasks of soybean casein digest broth for Bacillus; fluid thioglycollate for Clostridium.
3. Sporulation Medium (Nutrient Agar with 0.1% Manganese Sulfate)
4. 37°C or 55°C incubator
5. Sterile, cold DI water
6. Centrifuge tubes or bottles
7. Refrigerated Super-Centrifuge
8. 40% Ethanol (60 ml H_2O combined with 40 ml grain alcohol)

Procedure:

1. Inoculate tubes or flasks of the appropriate culture media from a stock culture or lyophilized culture of the spore forming organism. Consider this the primary inoculation.
2. Incubate tubes/flasks of a mesophilic Bacillus and Clostridium at 37±2°C; tubes of thermophilic Bacillus at 55-60°C.
3. Following incubation, check culture for purity via Gram's staining.
4. If the culture is determined to be pure, use the primary inoculation to inoculate a second group of tubes/flasks. Consider this the secondary inoculation. Incubate as in Step 2.

Item 3: Details on the SST neutralizer controls and the associated results

Per the SST Protocol, the Neutralizing Solution and Media are tested as a sterility control. A Neutralization Confirmation Control test is also routinely performed as part of the SST Protocol.

Neutralizing Solution (NS) and Media Sterility Control

A 0.5 ml aliquot of NS, a 0.5 ml aliquot of PBS, a 0.5 ml aliquot of saline—Tween (Wash Solution 1), and a 0.5 ml aliquot of saline (Wash Solution 2) will be individually cultured, incubated, and visually examined for growth. The acceptance criterion for this control is lack of growth.

Neutralization Confirmation Control

The neutralization of the test substance will be performed by exposing an uninoculated, test substance as in the test. Following the light exposure, the test substance in the Petri dish will be neutralized with 800 µl NS. Mix NS with the liquid inside the dish. After 30 seconds, an aliquot of 100 µl sterile DI water containing low numbers of spores (approximately 100 spores) will be added to the dish. After 30 seconds, wash and collect all liquid from the dish as described in test procedure 11.4.13 to 11.4.15 (see excerpt below). Filter the solution in the same manner as the 10^0 dilution in the test, and plate the filter on the TSA plate. For the numbers control, spray an uninoculated sterile Petri dish (or Aluminum

weighing dish) with Wash Solution 2 (0.85% NaCl) followed by light exposure. The steps of neutralization, adding spores to the dish, washing and collecting liquid inside the dish, filtering and plating the filter, and incubating the plate will be the same as above. The acceptance criterion for this control requires the neutralization control and corresponding numbers control results to be within 0.5 log.

11.4.13. Add 3.4 ml NS Wash Solution 1 to the Petri dish (or 8.4 ml Wash Solution 1 to the Aluminum dish); scrape the bottom of the plate with a cell scraper, resuspend the solution with a pipette.

t t.4. t4. Collect all of above solution into the corresponding tube marked 10^{0}.

t1.4.15. Add another 5 ml of Wash Solution 2 onto the same Petri dish plate and resuspend the solution as above (no need to use cell scraper). Collect this 5 ml solution into the same tube marked with 100, mix the solution thoroughly (after two washes, this $t0^{0}$ tubes should contain 10 ml of solution). Note: Skip this step for Aluminum dishes. The $t0^{0}$ dilution tube should contain 10 ml of solution after Step t1.4.14.

<u>Amendment to protocol (for Tests performed in February-March 2008)</u>

Aliquots of NS Wash Solution t and buffer Wash Solution 2 are to be adjusted according to whether Catalase C100 or C30 (NS made with C30 shown as [] bracketed amounts) are used to make the NS Wash Solution 1. Aliquots of NS Wash Solution t in Step 1 t.4.13 are 0.5 [t.0] ml (glass Petri dish) and 0.8 [t.6] ml (Aluminum dish) and the total additional Wash Solution 2 after two washes in Step 1 t.4.15 is 9 [8.5] ml (glass Petri dish) and 8.4 [6.8] (Aluminum dish), so that the final volume in each dish is approximately 10 ml.

<u>Neutralization Confirmation Control results for SST data previously submitted to the Agency</u>

Results of the Neutralization Confirmation Control were included with the study data for trials performed on Feb. 27, 2008 to March 5, 2008, and submitted on March t7, 2008, in Volume 2 of 2, Efficacy & Method Validation Report (MRID # 4737410 t), Appendix t, pages 40 and 4 t. However, the labels for these control data were inadvertently deleted from the presented data, and Clean Earth Technologies, LLC, apologizes for the omission of these labels. The relevant Neutralization Confirmation Control data can be found on the 5th and 6th lines of each of the data group by lot and organism. The summarized efficacy results for these studies are found on page t4 of the above referenced report. Neutralization Confirmation Control data for Bs on glass carriers with Peridox Use Dilution form batch CET062705S016, tests performed on July 27, 2005, and for aluminum carriers with the same batch and performed on August 18, 2005, were not included in Appendix 1 of the cited report. The Neutralization Confirmation Control results are presented in the following table:

Organism	Batch	Carrier	Number Control (T0) [CFUs]	Neutralization Control (T1) [CFUs]	Log (T0) –Log (T1) [pass ≤ 0.5]
Bs	CET062705SI016*	Glass	100	101, 102**	0.006
	CET103007S141	Glass	24	73	0.48
	CET062705SI016*	Aluminum	97	92, 103**	0.002
	CET103007S141	Aluminum	102	113	0.04
Bg	CET103007S143	Glass	50	87	0.24
	CET103007S142	Glass	78	97	0.09
	CET103007S142	Aluminum	72	79	0.04
	CET103007S143	Aluminum	67	84	0.10

*Batch number previously reported as CET1092904S100
**Neutralization Control performed twice.

Because of the unavailability of Sigma Catalase C100, an SST Protocol modification was made to permit the use of Catalase 30 as an alternative. The C30 has ½ the catalase concentration of the C100, so twice the amount of neutralizer solution (NS) made with C30 is needed in comparison with NS made with C100. To confirm this, a neutralization test was performed. The test results were number control = 74 (glass) and 87 (Aluminum) and neutralization control = 76 (glass) and 79 (Aluminum), so the | Log(T1) – Log(T0)| = 0.01 (glass) and 0.04 (Aluminum), which are < 0.5. This demonstrates that the use of C30 leads to satisfactory neutralization.

<u>Item 4: Details of sporulation media and spore quality control indicators used in the comparative study reported on page 14 of the previously submitted Efficacy & Method Validation Report</u>

Bg (Pine Bluff strain) was validated by gas chromatography-fatty acid methyl ester (GC-FAME) analysis at Midi Laboratories. Bs (ATCC 19659) has been validated by fatty acid analysis and library matching at Midi Laboratories. Spore suspensions were prepared by heat shock, washing of spore stock, and dilution to target titer density. Quality control indicators consisted of titer population determination and a purity control comprising streaking/plating of the suspension and examination to confirm the presence of a pure culture of the test organism, and by microscope inspection of stained spores prior to use of the preparation as inoculum. Protocols for washing and staining were included.

<u>Spore Cleanup</u>

Procedure used for spore suspension lots used for SST Studies at CET until February 2007, including SST data from tests conducted on July 27 and August 18 of 2005, and provided with the submission of March 13, 2008. (This procedure involves a 1% phenol wash).

Spores in stock may exhibit stickiness. There is a need to cleanup spore suspension and remove debris, unviable spores, any vegetative live/dead cell debris, and to purify/clean up the spore prep so that very little matrix is left. The resulting aliquots are more pure, and render highly concentrated viable spore prep.

Procedure

1. Aliquot 1 ml stock of Bg spores to 4 1.5 ml sterile centrifuge tubes, from large stock container (Battelle, Lot 121503 Bg, original titer ~5 x 10^{10} spores/ml, lot was very dirty with media and debris when received).
2. Vortex tubes 1 minute, then sonicate tubes 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution).
3. Add 1ml of 1% phenol (v/v) to each tube. Vortex tubes until pellet resuspended then for 30 seconds, then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Repeat phenol washings two more times, then pipette out third phenol wash from pellets.
4. Add 1ml of Ultra-Pure sterile water to each tube. Vortex tubes until pellet is resuspended for 30 seconds. Then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Only one washing for this step, to remove the majority of the phenol before the Na_2EDTA washing.
5. Add 1ml of 13mM Na_2EDTA to each tube. Vortex tubes until pellet is resuspended then for 30 seconds, then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Repeat Na_2EDTA washings two more times, then pipette out third phenol wash from pellets.
6. Add 1 ml of Ultra-Pure sterile water to each tube. Vortex tubes until pellet is resuspended for 30 seconds, then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Vortex until pellet resuspended then for 30 seconds, then sonicate 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Repeat Ultra-Pure sterile water washings two more times, then pipette out third phenol wash from pellets.
7. Add 0.2 ml Ultra-Pure sterile water to each Battelle tube. Vortex each tube until pellet is resuspended, then for 30 seconds. Combine all four Battelle tubes into one new tube.
8. Vortex each tube before titering 10 µl from each tube two times and dilute each series to 10^{-8}, and plate out 10 µl of 10^{-8} to 10^{-5} onto ¼ sectored LB agar plates (plate out each dilution series twice, 8 plates total).
9. Make spore stain (SOP 0072) slides from each tube and from stock Bg containers to compare cleanliness of preps under the microscope at 1000X and 2000X magnification (record pictures).

Spore Cleanup

Procedure used for spore suspension lots used SST Studies at CET between February 2007 and November 10, 2008, including SST data from studies conducted February-March 2008 and provided with the submission in March, 2008. (This procedure involves a 0.1% phenol wash).

1. Aliquot 1 ml stock of Bg spores to 4 1.5 ml sterile centrifuge tubes, from large stock container (Battelle, Lot 121503 Bg, original titer ~5 x 1010 spores/ml, lot was very dirty with media and debris when received).
2. Vortex tubes 1 minute, then sonicate tubes 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution).
3. Add 1ml of **0.1%** phenol (v/v) to each tube. Vortex tubes until pellet resuspended then for 30 seconds, then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Repeat phenol washings two more times, then pipette out third phenol wash from pellets.
4. Add 1ml of Ultra-Pure sterile water to each tube. Vortex tubes until pellet resuspended then for 30 seconds. Then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Only one washing for this step, to remove the majority of the phenol before the Na_2EDTA washing.
5. Add 1ml of 13mM Na_2EDTA to each tube. Vortex tubes until pellet resuspended then for 30 seconds, then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Repeat Na_2EDTA washings two more times, then pipette out third phenol wash from pellets.
6. Add 1 ml of Ultra-Pure sterile water to each tube. Vortex tubes until pellet is resuspended then for 30 seconds, then transfer re-suspension to new tube and discard tube with portion of pellet that cannot be resuspended. Sonicate new tube for 1 minute. Vortex until pellet resuspended then for 30 seconds, then sonicate 1 minute. Centrifuge tubes at speed 6 for 2 minutes. Pipette out supernatant of each tube (place in beaker with 4% Peridox Use Dilution). Repeat Ultra-Pure sterile water washings two more times, then pipette out third phenol wash from pellets.
7. Add 0.2 ml Ultra-Pure sterile water to each Battelle tube. Vortex each tube until pellet resuspended then for 30 seconds. Combine all four Battelle tubes into one new tube.
8. Vortex each tube before titering 10 µl from each tube two times and dilute each series to 10^{-8}, and plate out 10 µl of 10^{-8} to 10^{-5} onto ¼ sectored LB agar plates (plate out each dilution series twice, 8 plates total).
9. Make spore stain (SOP 0072) slides from each tube and from stock Bg containers to compare cleanliness of preps under the microscope at 1000X and 2000X magnification (record pictures).

Spore Cleanup

Procedure used on November 10, 2008 for spore suspension lots to be provided to ATS Labs for SST Studies. This procedure replaces the phenol wash with an ultra-pure sterile water wash.
Bs Spores (ATCC 19659), lot 101904BS from Presque Isle Cultures, stored in ethanol (percentage ethanol not given with certificate of analysis). Original titer @ 1×10^{10} spores/ml, ~60 ml quantity.

Spores stock tittered on 11/10/08, @ 8.2×10^{9} spores/ml, spores possibly stick and/or significant germinated (some dead after germination).

Aliquoted 6 ml stock spores to sterile 50 ml conical tube, performed cleanup as follows (11/12/08):

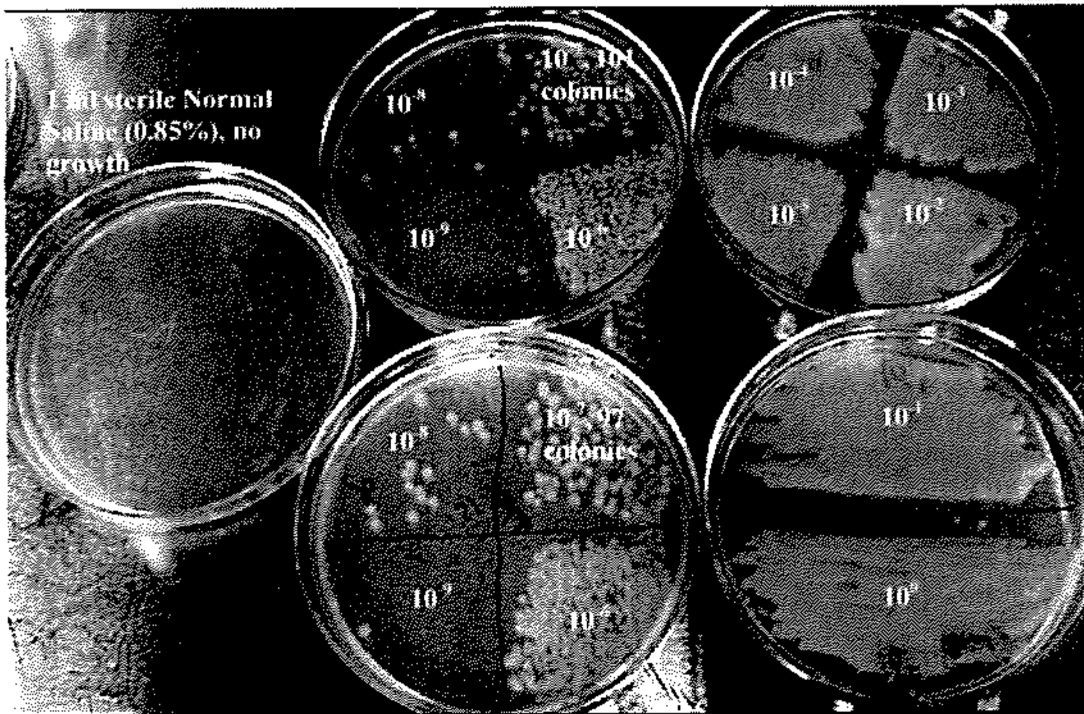
1. Centrifuged @ 10000 RPM for 5 minutes in large high-speed centrifuge.
2. Pipetted out supernatant.
3. Added 10 ml of Ultra-Pure sterile water (Wash 1).
4. Re-suspended pellet by vortexing and then vortexed for an additional minute.
5. Centrifuged @ 10000 RPA for 5 minutes.
6. Repeated Steps 2-5 two more times for a total of 3 washes.
7. After 3rd wash, centrifuged @ 10000 RPM for 5 minutes and then pipetted out supernatant and added 6 ml of Ultra-Pure sterile water.
8. Re-suspended pellet by vortexing and then vortexed for an additional minute.
9. Titered spores by dilution and plated out on KB agar to enumerate.
10. Heat shocked spores in water bath @ 60°C for 30-60 minutes.
11. Vortexed tube for 1 minute and then titered spores by dilution and plated out on LB agar to enumerate.
12. Centrifuged @ 10000 RPM for 5 minutes and then pipetted out supernatant and added 3 ml of 40% ethanol.
13. Vortexed until resuspended and then vortexed for an additional minute.
14. Transferred all of suspended spores to a sterile 14 ml tube with snap cap.
15. Vortexed 1 minute, then titered spores by dilution and plated out on LB agar to enumerate.
16. Sealed 14 ml tube to second snap on cap and parafilmed tightly. Stored tube @ 4°C.
17. Incubated LB agar plates overnight @ 37°C.
18. Next day, recorded colony numbers and calculated titers (colony numbers/ml plated x dilution factor = spores/ml)

Results of spore suspension titers to be shipped to ATS Labs (11/13/08) 10 ml, 10 µl plated):

	Dilution			Calculation spores/ml
	10^{-6}	10^{-7}	10^{-8}	
Colonies—Pre-heat Shock	88	7	0	8.8×10^{9} spores/ml
Colonies—Post-heat Shock	76	9	2	7.6×10^{9} spores/ml
Ethanol suspension	113	17	0	1.13×10^{10} spores/ml
Titer percent CFU number loss from heat shock			13.6%	
Total percent tier CFU loss from titer (11/10/08) after washings and heat shock			31.1%	

Recent spore suspension titer density determination results:

Bs spore CET Stock (from Presque Isle Cultures, lot 101904BS) titer and streaks
Titer of Bs spore stock (from Preque Isle Cultures, lot 101904BS), titered January 6, 2009, dilutions 10^{-9} to 10^{0}, 1 day incubation (photo below).



Day 1

Titer	Dilution			
	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1-	TNTC	101	5	1
2-	TNTC	97	15	1

Day 2

Titer	Dilution			
	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1-	TNTC	101	5	1
2-	TNTC	97	15	1

Item 6: Empirical evidence to support the use of a hand-held sprayer in the SST instead of the specialized wand—Additional information

A comparison of hand-held sprayers and the EDS Spray Wand was submitted to the Agency on October 3, 2008. Subsequently the Agency approved the use of the hand-held sprayer for the conduct of the SST studies. ATS Labs conducted the SST study on December 29, 2008. This study provided data reported as an enclosure with this submission. Peridox with EDS tested on aluminum carriers gave 7 logs of killing for each of three lots of Peridox all of which were greater than 2 months old, and one of which was 9 months old. Killing ≥ 6.5 logs was achieved on glass carriers for two lots, however for the oldest lot (CET032708S165) on glass carriers, only 5.8 logs of killing resulted. As the CET032708S165 on glass carriers was the first lot and carrier type tested, it was hypothesized that the spraying of the test liquid may not have thoroughly covered the inoculum. This was based on the observation in operator training in test procedure that consistent spraying of the glass Petri dish carriers is difficult and requires operator practice to attain proficiency. Further, given the excellent efficacy obtained on aluminum carriers, the results on the glass carrier for the one lot seemed anomalous. Further, it was found that the glass Petri dishes are not flat-bottomed. The dishes have a convex upward shaped bottom, (i.e., the center of the bottom of the dish is higher than the bottom near the dish wall by about 0.5 mm). This means there is an annular depression near the dish wall. Thus, the applied liquid tends to flow rapidly outward leaving a thinner coating of test liquid on the inoculated area than for a flat bottom dish. Still further, with the very low relative humidity (RH) in the laboratory at ATS Labs, typically RH <15%, evaporation is faster than for nominal test conditions at CET, which are ≥ 30%. A retest of the CET032708S165 lot on glass carriers with the spraying instructions amended to allow an extra spray pump actuation of the hand-held sprayer to account for the very low relative humidity subsequently resulted in > 6.6 logs killing. The combined result of the two tests of this lot on glass carriers is > 6.2 logs killing. The Instruction Manual has been revised in light of these results to that the direction for spray application of the Peridox Use Dilution and illumination now direct:

"After a contact of at least 3 minutes, proceed to Step 2, which is the illumination of the wetted surface by the EDS Light Wand at a distance of less than two (2) feet from the surface and with a motion across the surface at a rate of not more than one 91) foot per second. The surface after applying the 4% Use Dilution solution must remain wet for the entire time prior to illumination. Reapply 4% Use Dilution by spraying as necessary to maintain the wet surface. Illuminate the wet surface within ten (10) minutes of the spray application."

VI RESULTS

Test Substance	Carrier Type	Geometric Mean CFU/Control Carrier	Geometric Mean CFU/Test Carrier	Percent reduction (Log_{10} reduction)
Peridox Lot# CET032708SI65	Glass Petri Dish	7.94×10^7	1.29×10^2	>99.999
	Aluminum Dish	6.17×10^7	<6	>99.99999
Peridox Lot# CET050808SI70	Glass Petri Dish	7.94×10^7	2.51×10^1	>99.9999
	Aluminum Dish	6.17×10^7	<5	>99.99999
Peridox Lot# CET090308SI84	Glass Petri Dish	7.94×10^7	2.51×10^1	>99.9999
	Aluminum Dish	6.17×10^7	<3	>99.99999

Test Substance	Carrier Type	Geometric Mean CFU/Control Carrier	Geometric Mean CFU/Test Carrier	Percent reduction (Log_{10} reduction)
Peridox Lot# CET032708SI65	Glass Petri Dish	4.90×10^7	$<1.3 \times 10^1$	>99.9999%

VII AGENCY'S COMMENTS

(compiled April 16, 2009)

Technical Review

Appendix A – Supplemental Information

- Per the Agency's request, the registrant provided the details for the sporulation medium for *Bacillus subtilis*. Nutrient agar amended with manganese sulfate was utilized; cultures were incubated for 5 days at 37°C with 95% sporulation achieved. The details in the procedure were found to be acceptable.

- Per the Agency's request, the registrant provided information on the neutralization confirmation control. Although the information on the methodology is adequate (see page 7 of Appendix A), it is not apparent if the volume of test chemical used in the neutralization assay (8 full pumps for glass and 15 half pumps for aluminum) is relevant to the treatment volume used in the efficacy test – this issue requires clarification by the registrant.
- In addition, it should be noted that the spray distance specified in the neutralization assay is listed as 2-2.5 inches – this is significantly different from the recommendation (5 inches) provided by the Agency in the November 18, 2009 technical review memorandum. The use of a spray distance of 2-2.5 inches instead of 5 inches must be justified by the registrant. Does the spray distance impact the volume sprayed, distribution of the test chemical and the outcome of the efficacy test?
- For the neutralization assay, the registrant must clearly define how the contact time was tracked (e.g., an official calibrated timer) and when it official began (i.e., beginning of the first spray or at the end of the last spray). The time elapsed between the initial pump to the final pump and associated ¼ turn rotations of the plates may be significant (e.g., 10-20 sec).
- The neutralization data provided in Appendix A supports the neutralization data provided in the efficacy reports.
- On page 15 of Appendix A, CET discusses the impact of the convex shape associated with the bottom of the glass Petri dishes. The registrant also claims that the spraying instructions were amended to allow for an extra spray actuation of the hand-held sprayer to account for very low humidity – CET must provide more details on what aspects of the spray application were changed to accommodate the shape of the Petri dish and the low humidity. Lot CET032708S165 was retested as a consequence of these observations – the first test yielded a $\log_{10}$ reduction (LR) of 5.79. The retest of lot CET032708S165 was carried out with "an extra spray actuation" to account for the low humidity and yielded a LR of 6.6. *Note*: It should be pointed out that the efficacy testing of subsequent lots was carried out with "a minimum of 9 forceful full-stroke spray actuations" for glass and "16 forceful half-stroke pump spray actuations" for aluminum.

Volumes 2 & 3 – Final **Study Reports**

Overview

- ATS labs performed the efficacy evaluations; the overall methodology is clearly described.
- Test conditions included the use of *Bacillus subtilis* (ATCC 19659) as the test microbe (spores), 3 minute contact time, use-dilution of the product in 200 ppm AOAC synthetic hard water, and the addition of a three-part organic burden to the test spore suspension.
- The efficacy method is a modification of ASTM Standard Quantitative Carrier Disk Method; the method is referred to as the Surface Sterilization Test (SST).
- The registrant provided the source and catalog number for the hand-held sprayer used to treat the test carriers.

Study Materials

- The test system was listed as *Bacillus subtilis*, ATCC #19659. TSA with Sheep Blood was designated as the recovery medium. Presque Isle Cultures generated the test spore suspension with nutrient agar amended with manganese sulfate as the sporulation medium.
- A combination of Dey Engley Broth and bovine catalase was used as the neutralizer agent.
- A three-part organic burden was added to the test spore suspension per the ASTM procedure.
- Hard water was specified as 200 ppm AOAC synthetic hard water – the actual titration was 202 ppm.
- *The Test Materials are appropriate for this study and no further discussion is required.*

Test Method

- The test spore suspension was diluted to approximately 5 × 10^8 spores/mL. The three part organic burden was added to the inoculum according to the ASTM procedure.
- The carriers were 60 mm glass Petri dishes and aluminum weighing dishes – each type was marked with a permanent marker to highlight the inoculation zone (3.5 cm diameter circle). Ten carriers of each type were used per lot tested.
- A sterile loop was used to spread the inoculum (100 µL) over the carrier surface (the 3.5 cm diameter circle). The inoculated carriers were dried at room temperature for one hr under vacuum and held at room temperature until used for testing. *Note*: If present, the presence of viable vegetative *B. subtilis* cells may inflate the counts – *MLB recommends holding the inoculated carriers overnight to reduce any potential impacts of vegetative cells on carrier counts. The Agency requests that the registrant provide the purity data (% spores) for the test spore suspension.*
- A use-dilution of 1 part Peridox to 5 parts 200 ppm hard water was prepared. The test substance was used within three hr of preparation. The spray bottles were sterilized prior to use.
- The neutralizer, Dey Engley broth and bovine catalase, was prepared and held at 2-8°C until used in testing.
- Primed sprayers were used to treat 10 dried inoculated carriers of each type. Three additional carriers were used for control counts. A minimum of 9 forceful pumps and 16 forceful pumps were used to treat the glass and aluminum carriers, respectively. Between pumps, the plates were rotated by ¼ turn. The spray was applied at a distance of 2-2.5 inches from the carrier surface. After the spraying, the analyst checked the plates to verify that the entire inoculated surface was thoroughly wet. *Note*: In a previous technical review, the Agency recommended that the application be made at a distance of 5 inches with 8 pumps. *The registrant must justify the use of the additional pumps and the shorter distance from the carriers.* The label indicates that a "thin layer" of product must be applied for the entire contact time. *Thus, was a thin layer maintained on the treated surfaces with the amount of product applied to the carrier surface?*

- *The report does not indicate how the contact time was tracked and if the contact begins with the first spray or the last spray – the registrant must address these issues.*
- After spraying, each dish was held at room temperature for 3 minutes before being exposed to the pulsed UV light system with a fluence of 28-33 mjoules/cm^3. *The Registrant must clearly identify and describe the UV light system used in this study (i.e., proto-type or final commercial product). Also, the wavelength range described in the draft label (200-850 nm) was not indicated in the testing protocol – the registrant must verify the wavelength of the UV used in the efficacy study.*
- It is important to note that room temperature was 19°C and the relative humidity was 12%.

Test System Recovery

- Neutralizer was added to the treated plates after the contact time; 1.2 ml added to the glass plates and 2.00 mL added to the aluminum plates. *The registrant must clearly define the parameters for conducting the neutralization confirmation in relation to the volume of neutralizer added to the treated plates in the efficacy component of the study.*

Culture/Incubation/Observation

- 0.45 µm filter units were used for recovery/plating on TSA with blood. The filters were incubated on media for up to five days at 35-37°C. Confirmation testing was performed on representative colonies.

Controls

- Purity controls were conducted on the spore preparation, organic burden, carriers, and the neutralizer solution.

Neutralization Confirmation Control

- The final protocol used in the final efficacy evaluation for neutralization is vague. The acceptance criterion stated in the protocol indicates a 1 log difference; however, 0.5 logs is indicates in Appendix A as the acceptance criterion. *The registrant must clearly describe the neutralization confirmation study used in the efficacy evaluation.*

Carrier Population Control

- Sterile saline, instead of the product, was applied to each control carrier dish using a supplied spray bottle. Neutralizer solution was applied to the control carriers following the exposure period and the inoculum was recovered per the method used for the treated carriers. The acceptance criterion for the control carriers was a minimum of 1×10^7 CFU/carrier.

Data Analysis

- Calculations were provided for measuring the initial test suspension, the geometric means of surviving spores on test carriers, log reduction, and percent reduction.

Analysis and Conclusions

- Control counts ranged from 6.2×10^7 to 7.9×10^7 CFU/carrier.
- Lot CET090308S184 demonstrated log reduction of 6.5 and 7.4 on glass and aluminum, respectively.
- Lot CET050808S170 demonstrated log reduction of 6.5 and 7.1 on glass and aluminum, respectively.
- Lot CET032708S165 demonstrated log reduction of 5.8 and 7.0 on glass and aluminum, respectively. This lot on glass was retested and resulted in a log reduction of 6.6.
- *It was not apparent which of the three lots was at least 60 days old, thus the registrant must identify this lot.*

Conclusions

Overall, the test methodology is clearly presented; an independent verification of the test results would be possible if necessary. The data support the sporicidal efficacy (i.e., a sporicidal decontaminate with a minimum 6 log performance standard) of PERIDOX® with the EDS on a hard non-porous surface. The selection of *B. subtilis* spores as a surrogate of *B. anthracis* is appropriate for the test chemistry – this was covered in previous technical reviews. MLB has the following questions and issues that must be addressed in order to completely evaluate the validity of the results:

1. The Agency requests that the registrant provide the purity data (% spores) for the test spore suspension.
2. The registrant must justify the use of the additional pumps and the shorter distance from the carriers.
3. The report does not indicate how the contact time was tracked and if the contact begins with the first spray or the last spray – the registrant must address these issues.
4. The Registrant must clearly identify and describe the UV light system used in this study (i.e., proto-type or final commercial product). Also, the wavelength range described in the draft label (200-850 nm) was not indicated in the testing protocol – the registrant must verify the wavelength of the UV used in the efficacy study.
5. The registrant must clearly define the parameters for conducting the neutralization confirmation in relation to the volume of neutralizer added to the treated plates in the efficacy component of the study.
6. It was not apparent which of the three lots was at least 60 days old, thus the registrant must identify this lot.

VIII REGISTRANT'S RESPONSE NAD AGENCY'S FINAL COMMENTS

Per the registrant's letter, provided via email, the following responses were offered to address the Agency's concerns.

1. Agency's Initial Comments: The Agency requested that the registrant verify the use of the spore production methodology as indicated in the Appendix (page 2).

Registrant's Response: The protocol we used to produce Bg Spore lot# 121503. A summary of the production of this lot is as follows:

12/9/08: Prepared modG in fermentor
Grew 10 ml starter culture from 11:00 AM to 4:30 PM
Started seed culture at 4:30 PM and grew overnight

12/10/08: Inoculated fermentor with seed culture at 8:15 AM

12/12/08: Heat-shocked in-situ (7:51 AM -8:21 AM)
Enumerated pre- and post- heat shock to determine % sporulation
Harvested spores via continuous-flow centrifugation
Washed spores 2X and stored 2-8°C

12/15/08 Washed spores a third time
Enumerated final washed spore suspension
Labeled as lot# 121508 and stored 2-8°C

Bs Spore Production and Suspension Preparation—Presque Isle Cultures

The following information was provided by Dr. Richard Gammon of Presque Isle Cultures. This information comprises a general description of the spore production for Bacillus subtilis (ATCC 19659) for lot 101904BS spore suspension in aqueous ethanol.

Reference:

Bacillus subtilis (ATCC 19659) Spore Production

C.H. Shaffer, Jr. PRD 66. [IFO 13722] Sporicidal Test (AOAC Methods 966.04, 1990). Testing of antimicrobial hand washing formulations (ASTM E1327-90). (Medium 3 30°C) Shipped Freeze-dried.

Per Dr. Gammon:

B. subtilis is cultured on fortified nutrient agar with $MnSO_4$, incubated at 37°C for 5 days at which time 95% sporulation is achieved. Spores are harvested, centrifuged, and washed with cold sterilized deionized water. After 3 washes, the spores are suspended in 40% aqueous ethanol and tittered using a standard dilution method and plating of dilutions in TSA

Agency's Final Comments: CET's response indicates that spores of *Bacillus subtilis* were produced per the amended nutrient agar method. The method is appropriate for

conducting the efficacy test. The submitted spore production method(s) for *B. atrophaeus* is not relevant to the efficacy data. No further response is necessary.

2. Agency's Initial Comment: The Agency's requests that the registrant provide the purity data (% spores) for the test spore suspension.

Registrant's Response: The original spore prep was analyzed by microscopy and spore stain and estimated at 50-75% purity. This prep was then subjected to a heat shock and three subsequent washes, and then a second heat shock followed by resuspension in one-half volume of 40% ethanol. The loss of CFU (44.3% loss) following this treatment represented loss of vegetative cells. The final preparation used for the GLP testing was about 69 to 96% spores. The data are presented below:

Treatment	Colony Counts for Dilution Series (0.01 ml plated)			Growth CFU/ml
	10^{-6}	10^{-7}	10^{-8}	
Initial	88	11	2	8.8×10^9
1st Heat Shock	79	11	2	7.9×10^9
2nd Heat Shock	71	9	0	7.1×10^9
40% Ethanol Suspension in ½ original volume	98	13	2	9.8×10^9
Total percent CFU loss $100-[(9.8 \times 10^9/ml \times 5\ ml)(8.8 \times 10^9\ /ml \times 10\ ml)] \times 100$			44.3	

Agency's Comments: Is the supplied purity data for *B. subtilis* or *B. atropheus*? Please clarify by providing the name of the microorganism. Based on EPA's experience, if the registrant followed the amended nutrient agar method, the percent spore purity should have been extremely high (greater than 95%). CET's data shows 69-96% spore purity. The impact of spore purity on the outcome of the test is unknown; however, this reviewer believes the efficacy data remain valid.

Registrant's Final Response: The purity data supplied in the Appendix A of the January 2009 submission giving pre-heat titer, post-heat titer, and ethanol suspension titer are for *Bacillus subtilis* (ATCC 19659, lot 101904BS, from Presque Isle Labs, validated at Midi Labs) spore suspensions shipped to ATS Labs for the November 2008 – January 2009 tests. Spore clean-up procedures were given for both Bg (*Bacillus atrophaeus*), Pine Bluff strain, validated at Midi Labs) spores and *Bacillus subtilis* spores. Heat shock to kill vegetative cells and spore cleanup are routinely performed in the preparation of all spore suspensions.

Agency's Final Comments: The response provided is adequate. No additional information is required.

3. Agency's Initial Comments: The registrant must justify the use of the additional pumps and the shorter distance from the carriers.

Registrant's Response: The use of the additional pumps was explained in Item 6 of the Appendix A to the Cover letter of the January 23, 2009 submission to the Agency (item 6 is appended to this response). In the very low relative humidity (RH ~ 11-12%) in the test laboratory at ATS Labs. It was found that 9 full pumps for the glass carriers and 8-9 full pumps or 16 half-pumps for the aluminum carriers were necessary to obtain consistent coverage and maintain wetness of the inoculated area for the required contact time. Because of variability in spray deposition and evaporation rate, an additional spray of 2 was necessary on some carriers to maintain wetness as noted in the ATS Labs reports. In higher relative humidity (typically ≥ 30%), the number of pumps needed to maintain visible surface wetness for the contact time was 8 full pumps.

Agency's Comments: CET should have alerted EPA prior to making the modifications to the testing protocol. An error in the spray distance reported in an earlier submission was acknowledged by CET. A 5 cm distance seems close to the inoculated carrier; however, this does not invalidate the efficacy data. It is important to note that CET states that neutralization confirmation assay was performed exactly as efficacy test. Thus, EPA assumes the spray volume/deposition between the two assays is comparable. Does CET concur with this assumption?

Registrant's Final Response: CET concurs. Briefly, we can confirm that the neutralization control was performed using glass Petri dishes and the same sprayers used in the test. The spray was applied as in the test, neutralized using the same neutralizer and amount used to neutralize the glass Petri dishes as in the test. This material was collected, filtered, and then the organism was added. The use of the glass Petri dishes represented the theoretical worst case level of neutralization as it used less neutralizer then the aluminum dishes. The testing lab elaborated that the neutralization confirmation control was performed using the glass dish which had the least amount of neutralizer applied to it. It was done exactly as in the test, i.e., the carriers were sprayed using the same sprayers as the test. Then the same volume of neutralizer as applied to the test carriers (1.20 ml) was added to the dish. It was tilted to neutralize the entire volume of test substance and then 3.0 ml of saline Tween (Wash Solution 1) was added. This volume was aspirated from the dish and transferred to a sterile tube. The dish was rinsed a second time with 5.0 ml of Wash Solution 1. The rinse solution was transferred to the test tube and then the mixture was filtered and rinsed. The organism suspension was added to the filter, evacuated and then the filter was plated on blood agar. Both neutralizer confirmation control sections indicate that the neutralization confirmation control treatment was performed using the glass Petri dishes were neutralized and filtered as the 10^0 dilution in the test.

Agency's Final Comments: The response provided is adequate. No additional information is required.

4. Agency's Initial Comments: The report does not indicate how the contact time was tracked and if the contact begins with the first spray of the last spray—the registrant must address these issues.

Registrant's Response: For test 1 (3 lot study A07187) the contact time was tracked using 2 calibrated timers which were started at the same time. The contact time began with the last spray and prior to verification that the carrier was completely wet and was completed as the carrier was being flashed. Only one carrier needed an additional spray (paragraphs 4-6 on page 11). For test 2 (lot study A07226) the contact time was tracked using 1 calibrated timer. The contact time began with the last spray and prior to verification that the carrier was completely wet. In addition, all carriers were re-observed between 1-2 minutes into the exposure time. Additional spray(s) were applied as needed. The exposure time was not extended for carriers needing additional sprays. The exposure time was completed as the carrier was being flashed (paragraphs 1 and 2 on page 11).

The time to spray the carriers is estimated at approximately 10-15 seconds for the glass carriers and approximately 10-20 seconds for the aluminum dishes.

Agency's Final Comments: The response provided is adequate. No additional information is required.

5. Agency's Initial Comments: The registrant must clearly identify and describe the UV light system used in this study (i.e., proto-type or final commercial product). Also, the wavelength range described in the draft label (200-850 nm) was not indicated in the testing protocol—the registrant must verify the wave length of the UV used in the efficacy study.

Registrant's Response: The UV light system used in the efficacy study is a commercial production system with the addition of a Light Wand external housing mounting that holds the Light Wand a fixed distance above the carriers to be exposed. Consequently, the spectrum is identical to that of the production embodiments. Light output spans the spectral range of 200-850 nm. However, contribution to microbicidal efficacy is principally by photons with wavelength below 400 nm, and while some DNA destruction or irreversible impairment is caused by photochemical mechanisms for photon energy less than about 4 eV (wavelength longer than 320 nm), predominant photochemical kill is driven by more energetic photons, i.e., light with wavelength below 320 nm), the predominant photochemical kill is driven by more energetic photons, i.e., light with wavelength below 320 nm. Representative spectra and the spatial light pattern are shown below.

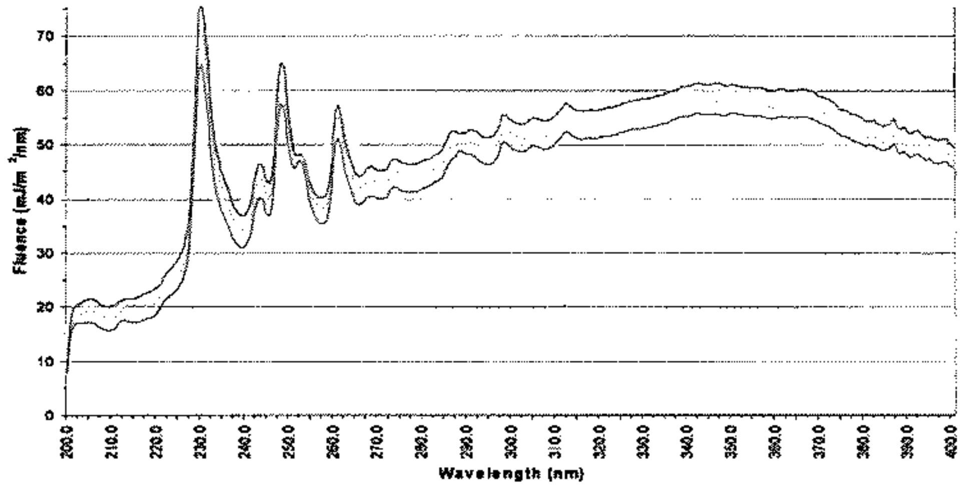


Fig. 1. Output spectrum of EDS Light Wand shows ± 5% variability over a 3 hour period.

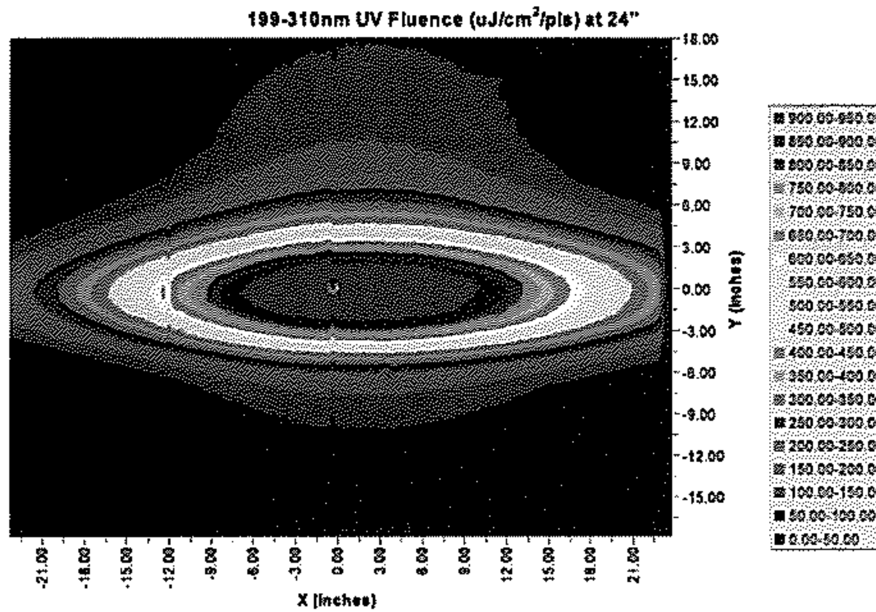


Fig. 2. The spatial distribution of UV (199-310 nm) fluence (μJ/cm²/pulse) for the EDS Light Wand with a standoff distance of 24 inches. Note that the pattern scales similarly with standoff distance.

Agency's Final Comments: The response provided is adequate. No additional information.

6. Agency's Initial Comments: The registrant must clearly define the parameters for conducting the neutralization confirmation in relation to the volume of neutralizer added to the treated plates in the efficacy component of the study.

Registrant's Response: The neutralization confirmation control was performed using the glass dish to which the least amount of neutralizer had been applied. It was done exactly as in the test, i.e., the carriers were sprayed using the same sprayers as the test. Then the same volume of neutralizer as applied to the test carriers (1.20 ml) was added to the dish. It was tilted to neutralize the entire volume of test substance and then 3.0 ml of saline Tween (Wash Solution 1) was added. The volume was aspirated from the dish and transferred to a sterile tube. The dish was rinsed a second time with 5.0 ml of Wash Solution 1. The rinse solution was transferred to the test tube and then the mixture was filtered and rinsed. The organism suspension was added to the filter, evacuated, and then the filter was placed on blood agar. Both neutralizer confirmation control sections indicate that the neutralization confirmation control treatment was performed using glass Petri dishes and that the glass Petri dishes were neutralized and filtered as the 10^0 dilution in the test.

Agency's Final Comments: CET states that neutralization confirmation assay was performed exactly as efficacy test. No further response is required.

7. Agency's Comments: It was not apparent which of the three lots was at least 60 day old, thus the registrant must identify this lot.

Registrant's Response: They were all > 60 days old.

Agency's Final Comments: The response provided is adequate. No additional information.