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Evaluation of Analytical Methods for The Detection of *Bacillus Anthracis* Spores: Compatibility With Real-World Samples Collected From Outdoor And Subway Surfaces

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EVALUATION OF ANALYTICAL METHODS FOR THE DETECTION OF *BACILLUS ANTHRACIS* SPORES: COMPATIBILITY WITH REAL-WORLD SAMPLES COLLECTED FROM OUTDOOR AND SUBWAY SURFACES

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Disclaimer

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Questions concerning this document, or its application should be addressed to:

Dr. Worth Calfee
U.S. Environment Protection Agency
109 T.W. Alexander Drive
Mail Code: E343-06
Research Triangle Park, NC 27711
calfee.worth@epa.gov
919-541-7600

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Abbreviations and Acronyms

Acronym	Definition
B. a. Sterne	*Bacillus anthracis* Sterne
B. anthracis	*Bacillus anthracis*
BHIB	Brain Heart Infusion Broth
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological Safety Cabinet
°C	Degree(s) Celsius
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit(s)
Ct	Threshold Cycle
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
DOHMH	Department of Health and Mental Hygiene
EPA	U.S. Environmental Protection Agency
ERLN	Environmental Response Laboratory Network
FAM	Fluorescent reporter dye on 5' end of PCR probe (6-carboxyfluorescein); emits at ~517 nm
HVAC	Heating, Ventilation, and Air Conditioning
ID	identification
L	Liter(s)
µL	Microliter(s)
MCE	Methyl Cellulose Ester
min	minute
mL	Milliliter(s)
ModG	Modified G
NRF	National Response Framework
NTC	No Template Control
NYC	New York City
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline plus 0.05% Tween
PC	Positive Control
PCR	Polymerase Chain Reaction
PE	Performance Evaluation
pg	picogram
PMP	Paramagnetic Particle
PVDF	Polyvinyldiene Difluoride
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
QMP	Quality Management Plan
qPCR	quantitative PCR

Abbreviations and Acronyms (Cont.)

Acronym	Definition
rcf	relative centrifugal force
rpm	revolution(s) per minute
RV-PCR	Rapid Viability PCR
RWI	Real-World Interferent
SBA	Sheep Blood Agar
SOP	Standard Operating Procedure
T&E II	Testing and Evaluation II Program
TOCOR	Task Order Contracting Officer's Representative
TSA	Technical Systems Audit
TSB	Trypticase Soy Broth
VFC	Vacuum Filter Cassette
VIC	Fluorescent reporter dye on 5' end of PCR probe (emits at ~551 nm)

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EXECUTIVE SUMMARY

Under Emergency Support Function #10 of the National Response Framework (NRF), the United States Environmental Protection Agency (EPA) is responsible for the remediation of land and public infrastructure following a biological contamination incident such as an act of bioterrorism involving the release of *Bacillus anthracis* (*B. anthracis*) in an urban area. (NRF: https://www.fema.gov/media-library/assets/documents/117791). EPA, in coordination with other Government agencies, National Laboratories, and Stakeholders have conducted studies to support preparation for that role, which have included release of surrogates for *B. anthracis* spores in outdoor environments and subway stations to better understand the transport of aerosol releases and to assess models to predict spread of the particles. Further, EPA has developed analytical methods including microbiological culture, polymerase chain reaction (PCR), and Rapid Viability (RV) PCR quantification and identification protocols for *B. anthracis* species that are used by EPA's Office of Emergency Management Environmental Response Laboratory Network (ERLN) (Shah, 2017). EPA and the Centers for Disease Control and Prevention (CDC) have also developed and established surface sampling methods using a wetted Sponge-Stick™ (Rose et al., 2011) and a vacuum filter cassette (VFC) (Calfee, 2013), and associated organism recovery methods from those sampling media to be used with the developed culture and RV-PCR analytical methods.

Following a biological contamination incident, the spatial extent of the contamination should be determined using established sampling and analytical methods such as those noted above. Both surface sampling methods will also collect ambient particulate matter that has accumulated on surfaces to be sampled, along with the target organism when surface samples are taken. That ambient particulate matter can then be present in the sample extract used to identify the presence of *B. anthracis*. The collected and recovered ambient particulate matter represent potential real-world interferents (RWIs) to the EPA culture and molecular analysis methods and may adversely impact their ability to identify the presence of *B. anthracis* spores. EPA therefore seeks to assess the impact of potential RWIs (present in Sponge-Stick and VFC samples) on current culture and molecular analysis methods. Having an assessment of the impact, if any, will help EPA understand limitations of the current methods for contaminant spread and extent mapping and identify possible opportunities or needs for method improvement.

A surface sampling campaign was conducted in the mid-town Manhattan (Times Square and Grand Central Station areas) in November 2017 using Sponge-Sticks and VFCs to sample target surfaces representative of those of relevance to EPA for site remediation. The target surfaces were selected in coordination with EPA, New York City (NYC) Transit personnel, NYC Department of Health and Mental Hygiene (DOHMH), and local law enforcement. The surface samples collected contained material that would be representative of such matter that would also be collected if sampling for *B. anthracis*, post-bioterrorism incident. The surface sampling was not being conducted to characterize background organisms or to baseline whether a target organism was present. No such analyses were performed on the collected samples.

The surface samples collected from the field were sent to the analytical laboratory and stored in refrigerated conditions (2 to 8°C) until spiked with a known quantity of *Bacillus anthracis* Sterne (*B. a.* Sterne) spores (as a surrogate for other virulent *B. anthracis* strains) to apply a target of 30, 300, or 3,000 spores per surface sample. The spores were then recovered from the samples, along with any physically removed material previously collected from the surface sample and

subsequently analyzed to identify the presence of *B. a.* Sterne using EPA's culture and RV-PCR methods. The culture method used Sheep Blood Agar (SBA) as the growth medium. The RV-PCR method including PCR assays targeting chromosomal and a pXO1 plasmid genes was used with independent analyses of samples run for each assay.

The performance of the culture method was assessed by determining percent recovery efficiency of presumptive *B. a.* Sterne spores spiked onto the samples, which was also used to define frequency of false positives and false negatives. The performance of the molecular method was assessed by whether a positive identification was made, which was then used to determine a frequency of accurate identification, false positives, and false negatives.

The foremost conclusion is that the *B. anthracis* RV-PCR analysis method was very accurate (> 97%) in correctly identifying the presence or absence of *B. a.* Sterne in Sponge-Stick samples that had previously collected background material from real-world surface sampling. The culture method was less accurate (77%) in correctly identifying the presence or absence of *B. a.* Sterne in the same Sponge-Stick samples, meaning the presence of real-world material collected during surface sampling can hinder the culture method performance. The 18 field blank samples analyzed – 15 samples spiked with *B. a.* Sterne and 3 samples not spiked – were 100% accurately identified by the culture method.

Neither the culture nor molecular analytical methods performed as well with surface samples collected using the VFC compared to the Sponge-Sticks sampling method. The decrease in performance as measured by the accuracy of properly identifying the presence or absence of *B. a.* Sterne spiked onto the samples was attributed primarily to poor physical recovery of *B. a.* Sterne from the VFC methyl cellulose ester (MCE) collection substrate and, also, possibly, to the collected ambient particulate matter. It is possible that the spiking method (drops of a *B. a.* Sterne suspension applied directly onto the VFC collection substrate) affected the physical recovery of organisms for subsequent analysis. Collection of the *B. a.* Sterne as an aerosol while simultaneously collecting the ambient particulate matter (as would be the case in an actual sampling campaign following a bioterrorism incident) may yield improved recovery efficiencies. This effect, however, may be most apparent when there is an opportunity to collect much ambient particulate matter.

RV-PCR can be used to positively identify viable *B. a.* Sterne in presence of complex, dirty sample matrices from Sponge-Stick surface samples. The background flora and grime collected on the Sponge-Stick can impact the lower limit of detection and/or suppress the sensitivity of the *B. a.* Sterne signal, yet samples with as few as a nominal quantity of 15 *B. a.* Sterne spores could be positively identified in the presence of real-world background matter.

The RV-PCR method requires great care and diligence to implement effectively. Most notable, the method required changing gloves between procedural samples for each step, which is time-consuming. Glove changing is critical to avoid cross-contamination samples, which would negatively impact key decisions in the response, response timelines, credibility, and cost.

The results from this study will be useful to those analyzing samples collected following a bioterrorism incident. The study demonstrates that results from traditional culture-based methods may be confounded by an overwhelming presence of background flora, obscuring the presence of *B. anthracis* spores.

1.0 INTRODUCTION

1.1 Background

Under Emergency Support Function #10 of the National Response Framework (NRF), the United States Environmental Protection Agency (EPA) is responsible for the remediation of land and public infrastructure following a biological contamination incident such as a bioterrorism incident involving release of *Bacillus anthracis* (*B. anthracis*) in an urban area. (NRF: https://www.fema.gov/media-library/assets/documents/117791). EPA, in coordination with other Government agencies, National Laboratories, and Stakeholders have conducted studies to support preparation for that role, which have included releases of surrogates in outdoor environments and subway stations to better understand the transport of aerosol releases and to assess models to predict spread of the particles. EPA has developed culture, polymerase chain reaction (PCR), and Rapid Viability (RV) PCR quantification and identification protocols for *B. anthracis* for use by EPA's Office of Emergency Management Environmental Response Laboratory Network (ERLN) (Shah, 2017). EPA and the Centers for Disease Control and Prevention (CDC) have developed and established surface sampling methods using a wetted Sponge-Stick (Rose et al., 2011) and a vacuum filter cassette (VFC) (Calfee, 2013), and associated organism recovery methods from those sampling media to be used with the developed culture and RV-PCR methods.

Following a biological contamination incident, the spatial extent of the contamination should be determined using established sampling and analytical methods such as those noted above. Both surface sampling methods will collect ambient particulate matter that has accumulated on surfaces, along with the target organism when surface samples are taken. That collected ambient particulate matter can then be present in the sample extract used to identify the presence of *B. anthracis*. The collected and recovered ambient particulate matter represent potential real-world interferents (RWIs) to the EPA culture and molecular analysis methods and may adversely impact their ability to identify the presence of *B. anthracis* spores. It is therefore critical to assess the impact of potential RWIs (present in Sponge-Stick and VFC samples) on current culture and molecular analysis methods. Having an assessment of the impact, if any, will help determine limitations of the current methods for contaminant spread and extent mapping and identify possible opportunities or needs for method improvement.

1.2 Objective

The objective of this study was to assess the impact of RWIs collected on Sponge-Stick and VFC samples on the current EPA-recommended culture and molecular methods for identification of viable *B. anthracis* spores in environmental samples.

1.3 Scope

A surface sampling campaign was conducted in mid-town Manhattan (Times Square and Grand Central Station) in November 2017 using Sponge-Sticks and VFCs to collect particulate matter that had accumulated on selected surfaces and be representative of such matter that would also be collected if sampling for a target organism, post-bioterrorism incident. The surface sampling was not being conducted to characterize background organisms or to baseline whether a target organism was present. No such analyses were performed on the collected samples.

The surface samples collected from the field were sent to the analytical laboratory and stored in refrigerated conditions until spiked with *Bacillus anthracis* Sterne (*B. a.* Sterne) spores. The spores were then recovered from the samples, along with any physically removed material previously collected from the surface sample and analyzed to identify the presence of *B. a.* Sterne using EPA culture and RV-PCR methods. (Initially, samples were analyzed using the 2012 version of the analytical methods, which did not include an enrichment step, but then the enrichment culture step was added per the 2017 version (2nd Edition) of the EPA's Protocol for Detection of *B. anthracis* Spores from Environmental Samples During the Remediation Phase of an Anthrax Incident so that the analytical methods used here were as consistent as possible with the current EPA methods). The performance of the culture method was assessed by determining percent recovery efficiency of presumptive *B. a.* Sterne spores spiked onto the samples, which was also used to define frequency of false positives and false negatives. The performance of the molecular method was assessed by whether a positive identification was made, and that was then used to determine a frequency of accurate identification, false positives, and false negatives.

It is important to note that this study was not solely an assessment of the analytical method to identify *B. a.* Sterne in the presence of potential RWIs, but rather, the entire method end-to-end to include physical recovery from the filter media (and other grime or flora associated with the

filter's operation in its intended use) followed by the *B. a.* Sterne analytical method. It is that end-to-end analysis that was the key element to assess method performance. Consequently, the study provided information on the limitations and opportunity for improvement of the methods as well as providing a baseline of processing and analyzing samples that may be encountered in an actual incident response.

2.0 MATERIALS AND METHODS

2.1 Sampling Methods

Two sampling tools were used to collect samples form target surfaces: Sponge-Sticks and VFCs. EPA has established sampling protocols for both the methods, which were then summarized in work instructions for the field team to execute.

2.1.1 Sponge-Sticks

3M Sponge-Sticks™ pre-wetted with a neutralizing buffer (3M Part number SSL10NB) – shown in Figure 1 – were purchased for sample collection per established EPA sampling methods (EPA 2013 and Tufts et al., 2014) and CDC's Anthrax Surface Sampling Guide. The Sponge-Sticks were used to sample a 10 x 10-inch area (defined by a template overlaying the target surface) following the sampling pattern (30 linear passes over the area in a vertical, horizontal, and diagonal pattern) defined in the EPA sampling method.

Figure 1. Pre-Wetted Sponge-Stick from 3M Used for Surface Sampling

2.1.2 Vacuum Filter Cassettes

VFCs, 37-mm-diameter, 0.8 um pore MCE membrane (Part No. SKC 225-3-01) were purchased for surface sample collection per established EPA sampling methods (Calfee, 2013). An assembled and disassembled VFC are shown in Figure 2. The VFCs were used to sample a 12 x 12-inch area (defined by a template overlaying the target surface) over a 5-min (300-sec) sampling duration following the sampling pattern (50 linear passes over the area in a vertical S-pattern followed by 50 linear passes in a horizontal S-pattern, with each pass being ~3-sec duration) defined in the EPA method. A battery-operated personal sampling pump (Leland Legacy, SKC International, Eighty Four, PA), set to 10 liter/minute (L/min) sampling rate was used.

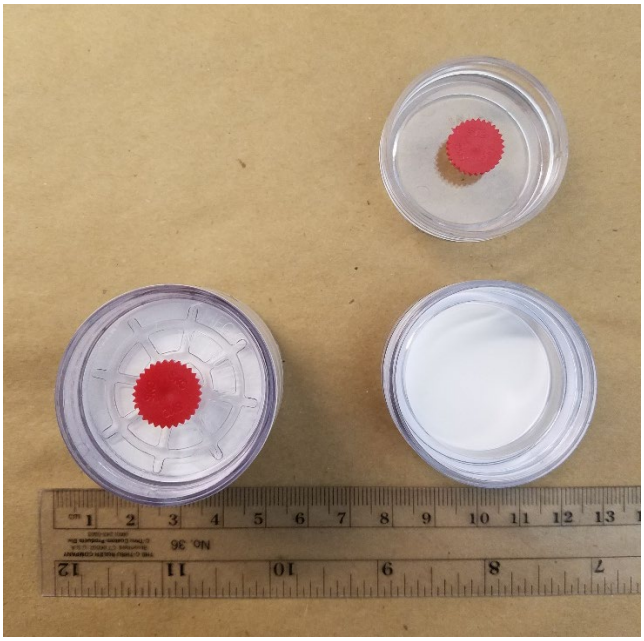

**Figure 2. Vacuum Filter Cassette (37-mm Diameter)
Assembled (Left) and Disassembled (Right) Used for Surface Sampling**

2.2 Sampled Surfaces

Both the Sponge-Sticks and VFCs were used to sample target surfaces in the NYC mid-town Manhattan area (Times Square and Grand Central Station). Representative images of the field team collecting surface samples with Sponge-Sticks and VFCs are shown in Figure 3 and Figure 4, respectively. The samples were collected following established EPA sampling procedures by a sampling team the week of 12 November 2017. Prior to the sampling campaign, planning, and coordinating meetings with the EPA Task Order Contracting Officer's

Representative (TOCOR), New York City (NYC) Transit personnel, NYC Department of Health and Mental Hygiene (DOHMH), and local law enforcement were held in NYC to define priority target surfaces and coordinate access to locations throughout the Times Square and Grand Central Station area. The primary rationale and guidelines used to target for sampling included:

- Location of relevance to EPA sites considered for site remediation.
- Having adequate surface area available to collect at least 18 samples (could also include multiple of the same surface to attain required surface area).
- At least 25% of sampling locations were to be sampled with VFCs.
- At least 50%, but not more than 75%, were to be below ground.
- Locations were dispersed within the Times Square and Grand Central Station vicinity.
- At least two surfaces were to be conducive to sampling with both methods.

Figure 3. Electronic Display Panels (Below Ground) Located in Times Square 42nd Street Station, Near Track 3 – Sampled with Sponge-Sticks

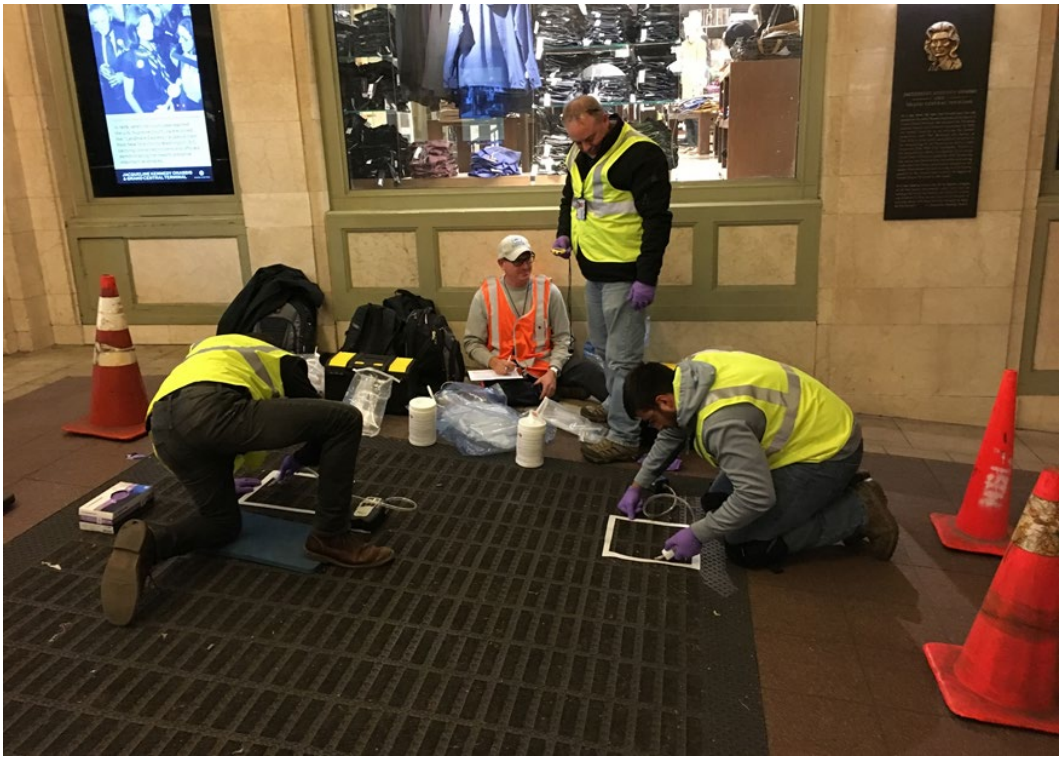

Figure 4. Carpet Surface Located by the Jackie O Entrance to Station, Off 42nd Street – Sampled with Vacuum Filter Cassette

In summary, 23 distinct surfaces were targeted consistent with the above characteristics that also were consistent with surfaces used during previous EPA sampling campaigns. Three (3) of those surfaces were sampled using both the Sponge-Stick and VFC methods for a total of 26 distinct surface/sampling method sets of samples. Field blanks were collected for each the Sponge-Stick and VFC samples, accounting for 2 of the 26 sample sets collected. The field blanks were samplers handled in the exact same manner as those samplers used to collect from surfaces, except that the field blanks never contacted a surface to actively collect a sample (i.e., opened and exposed to the collection environment, then packaged). They were packaged, shipped, and stored in the same manner as samples that had been used to sample a surface. Fourteen (14) of the surfaces were located below ground in areas associated with the subway system, with the remaining 12 surface locations above ground. Twenty (20) discreet samples were collected for each surface set comprising 18 samples for subsequent analysis (see Section 2.3) and 2 spare samples. A total of 520 samples were collected.

A brief summary description, including a photograph, of each target surface and location is provided in Appendix A.

2.3 Test Matrix

Each of the collected surface samples described in Sections 2.1 and 2.2 was spiked with *B. a.* Sterne spores, extracted, and the extract analyzed to quantify and identify recovered *B. a.* Sterne to assess the EPA-provided culture and RV-PCR methods.

The completed test matrices for the Sponge-Stick and VFC samples are given in Table 1 and Table 2, respectively. In total, 468 surface samples were analyzed, comprising 342 Sponge-Stick samples and 126 VFC samples.

The surface sample target and associated identifier (ID) used to uniquely name the samples collected are provided in the first two columns. In the surface ID, the suffix of "-A" or "-B" indicate whether the sample was collected above ground or below ground, respectively. The target spore load of 0, 30, 300, or 3,000 was the number of spores intended to be spiked onto the filter. Following physical extraction, the sample volume was split nominally in half to result in 0, 15, 150, and 1,500 *B. a.* Sterne spores available for each of the two detection methods (culture and RV-PCR). The method details are discussed in further detail in Section 2.4. Each sample set of 18 had 5 samples spiked with the target 30, 300, and 3,000 *B. a.* Sterne spores; the remaining 3 samples that were not purposely spiked (0-load condition) with *B. a.* Sterne served as negative controls. The field blanks served as a baseline to represent the expected best-case performance of the method because of the absence of potentially competing or interfering grime or flora.

Culture and RV-PCR analytical methods were used to detect and/or quantify recovered *B. a.* Sterne spores spiked and subsequently recovered in the sample extracts. Sheep Blood Agar (SBA) was the primary medium used for all culture analyses. Details regarding the analytical methods are discussed in Section 2.4.

Table 1. Test Matrix for Sponge-Stick Samples

Surface Sample	Surface ID	Target Spore Loads onto Filter[a]	Nominal Spores Available per Analytical Method[b]	Replicates	Analytical Method[c]	
					Culture	Molecular
Floor (Tile)	FLTILE-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Floor (Concrete)	FLCON-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Steps (w/Metal Grid)	STEPS-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Wall Tile	WLTILE-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Glass Window	GLSWIN-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Electrical Display Panel	EDPAN(B)-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Glass Panel	GLSPAN-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Fluor Light Fixture	FLLFIX-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Overhead Sign	OHSIGN-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Metro Card Machine	MCMACH-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Subway Car Filter Grille	SCGRIL-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Control (Field Blank)	FLDBLK-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Sidewalk Concrete	SWCON-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Electrical Display Panel	EDPAN(A)-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Crosswalk Signal	CWSIGN-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Telephone Booth	TELEBO-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Street Grating	STGRAT-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Crosswalk Painted	CWPNTD-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Granite Bench	GRNBEN-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR

(a) Target number of spores spiked onto filter – See Section 2.4.2 for discussion.

(b) Nominally half of the target quantity of spores loaded onto the filter were available for each of the two analytical methods – See Section 2.4.3 for discussion.

(c) SBA medium; RV-PCR assay, chromosomal and pXO1 gene targets.

Table 2. Test Matrix for Vacuum Filter Cassette Samples

Surface Sample	Surface ID	Target Spore Loads onto Filter[(a)]	Nominal Spores Available per Analytical Method[(b)]	Replicates	Analytical Method[(c)]	
					Culture	Molecular
Floor (Concrete)	FLCON-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Steps (w/Metal Grid)	STEPS-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Carpet/Rug	CARPET-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Subway Car HVAC Filter	SCFILT-B	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Sidewalk Concrete	SWCON-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Pavement (Asphalt)	PAVEMT-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR
Control (Field Blank)	FLDBLK-A	0/30/300/3,000	0/15/150/1,500	3/5/5/5	SBA	RV-PCR

(a) Target number of spores spiked onto filter – See Section 2.4.2 for discussion.
(b) Nominally half of the target quantity of spores loaded onto the filter were available for each of the two analytical methods spiked onto filter – See Section 2.4.3 for discussion.
(c) SBA medium; RV-PCR assay, chromosomal and pXO1 gene targets.

2.4 Microbiological Methods

All sample processing and analytical methods (both a culture and molecular analytical method) were conducted as described in the U.S. EPA Protocol for Detection of *Bacillus anthracis* in Environmental Samples During the Remediation Phase of an Anthrax Incident (EPA, 2012), with any differences or revisions noted. Toward the end of the analysis, the 2017, 2nd Edition (Shah, 2017) with Trypticase Soy Broth (TSB) enrichment broth procedure added to further assess the extracted Sponge-Stick or VFC for unrecovered *B. a.* Sterne. The methods were periodically reviewed with the EPA TOCOR and EPA method author to ensure proper application and successful transfer of the methods so that the results and observations of the method reflected those that could be expected when applied to field collected samples analyzed by the EPA's ERLN.

Following are sections that summarize specific procedures and steps applied to conduct the study.

2.4.1 Spore Bank

B. a. Sterne spores were used as the biological test agent for the entire study. This organism is a vaccine strain produced by Colorado Serum Company and is frequently used as surrogate to fully virulent *B. anthracis* strains such as Ames. The *B. a.* Sterne strain was handled as a Risk Group II agent following the Biosafety in Microbiological and Biomedical Laboratories (BMBL) guidelines and Battelle biosafety work practices for such agents. A spore bank was produced using sporulation broth as follows and used as needed for the duration of the study.

A cell bank of *B. a.* Sterne 34F2 prepared previously at Battelle from BEI Resources (BEI NR-1400) was used to grow an overnight culture on TSB. Isolated colonies were then used to inoculate 50 milliliter (mL) aliquots of nutrient broth and incubated overnight at 35 to 37 degrees Celsius (°C) with shaking at 200 revolutions per minute (rpm). Modified G (ModG) (500 mL) of sporulation broth (see Appendix B, Table 1 for formulation details) was inoculated with 50 mL of the overnight *B. a.* Sterne culture, and then incubated in a 3-liter (L) Fernbach flask at 35 to 37°C with shaking at 200 rpm. The culture was observed via wet mount microscopy every 1 to 3 days for sporulation. Following 5 days of incubation, the ModG culture reached > 99% sporulation. (Note, a spore bank was also prepared using Leighton-Doi medium [see Appendix B, Table 2 for formulation details], but spores from the ModG medium were used because > 99% sporulation was not achieved with Leighton-Doi and there was more cellular debris compared to spores prepared in the ModG medium.)

The sporulated culture was centrifuged at 10,000 relative centrifugal force (rcf) for 12 minutes in multiple 250-mL bottles. After removing and discarding the supernatant, the resulting pellets were resuspended to a total volume of approximately 100 mL with sterile distilled water (dH_2O), transferred into a sterile glass vessel, and heat shocked at 60 to 65°C for 1 hour in a water bath with gentle agitation. (Note: A control flask with a thermometer was used to ensure the desired temperature was achieved and maintained during the heat-shock step). The spores were then washed twice by repeated centrifugations at 10,000 rcf for 12 minutes using 100 mL dH_2O per wash. After the final centrifugation, the spores were resuspended to a total volume of 100 mL in sterile dH_2O. The spore bank was assigned a unique lot number and stored refrigerated at 2 to 8° C.

2.4.2 Spore Loading (Spiking)

Sponge-Sticks and VFCs were stored at 2 to 8°C prior to being spiked and processed (up to 16 months). All filter manipulations were performed within a surface-decontaminated, certified biological safety cabinet (BSC) and handled using sterile forceps and scissors.

B. a. Sterne spiking stocks were vortex-mixed and diluted using sterile dH_2O to the three spiking stock target concentrations shown in Table 3. Each spiking stock was spread plated onto SBA on the day of testing to calculate the actual concentration of spores spiked in colony forming units (CFU)/mL. The loading levels in Table 3 represent loadings that yielded enough *B. a.* Sterne spores in the sample recovery extracts to make meaningful measurements with both the culture and RV-PCR methods and covered a range that was expected to span their lower limit of detection or quantification, which was an important consideration to assess whether grime or flora associated with the samples affected the sensitivity or lower limits of the analytical method.

Table 3. Target *B. a.* Sterne Spore Loading Levels Spiked onto Each Sample Substrate

Loading Level	Stock Concentration (CFU/mL)	Target Total CFU per Sample[(a)]	Extract Volume (mL)	Theoretical Concentration in Extract (CFU/mL)
High	3.0×10^4	3,000	25	120
Medium	3.0×10^3	300	25	12
Low	3.0×10^2	30	25	1.2

(a) 100 microliter (µL) of stock suspension applied (20, 5-µL drops).

Each Sponge-Stick to be spiked with *B. a.* Sterne spores was positioned in a specimen cup so that the dirty side was facing up and 20 5-µL droplets, for a total of 100 µL of stock suspension, were pipetted onto the surface of each Sponge-Stick (the sides of the sponge that could contact the specimen cup wall were not spiked; see Figure 5). For VFCs, the final spiking stock concentrations were prepared in 50% ethanol and applied as 20 5-µL droplets distributed over the surface of collected particulates and filter by removing the plug from the port used to attach the nozzle and inserting the pipette tip with inoculum (see Figure 5). The spiked Sponge-Sticks were stored sealed in specimen cups at 2 to 8°C overnight and VFCs were dried overnight inside of a BSC (fan on) with red inlet plug removed.

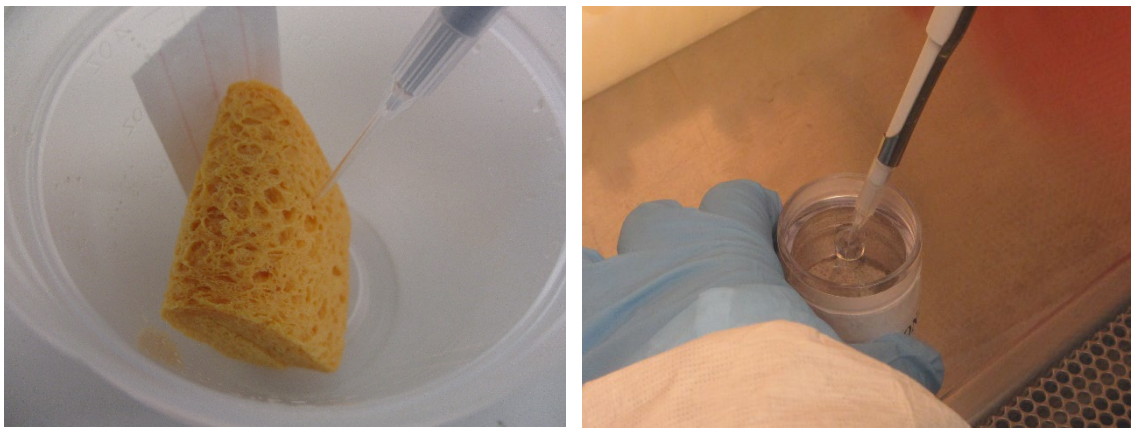

Figure 5. Sponge-Stick (left) and Vacuum Filter Cassette (right) After Spiking with the *B. a.* Sterne Suspension

2.4.3 Spore Recovery

Throughout the recovery procedure, gloves were changed between handling samples to limit the likelihood of cross-contamination between samples.

Sponge-Sticks

Following spiking, 90 mL cold (2 to 8°C) phosphate buffered saline (PBS) extraction buffer with 0.05% Tween 20 and 30% ethanol (final concentrations) was added into a Stomacher® bag. The remaining handle was removed, and the Sponge-Stick was unfolded and aseptically added to the Stomacher bag and homogenized for 1 minute at 260 rpm in a Stomacher (Seward). Each sample then sat for 10 minutes to allow foam to settle before removing the sponge. Absorbed liquid was expelled from the sponge into the Stomacher bag and the sponge was removed. A subset of the sponges (final 4 trials, 64 samples) were returned to the specimen cup and retained at 2 to 8°C for TSB enrichment. The remaining ~90 mL suspension was gently mixed by pipetting up and down three times with a sterile 50-mL pipet, then the suspension was split in half and centrifuged at 3,500 rcf for 15 minutes in a swinging bucket rotor at 4°C with the brake off. The pellets were suspended in ~25 mL of the supernatant to concentrate the sample. This pooled suspension was split in half and used for culture-based microbial analysis described in Section 2.4.4, and RV-PCR analysis as described in Section 2.4.5.

For Sponge-Stick TSB enrichment, the extracted sponge and remaining spore recovery suspension was saved at 2 to 8°C for the final four trials of analysis. Twenty-five mL of TSB was added to the saved sponge along with any remaining spore recovery suspension, then

incubated at 37 ± 2°C for 24 to 48 hours. Samples with turbid TSB broth were then streaked onto three SBA plates for isolation. Colonies with *B. a.* Sterne morphology that were isolated on these streak plates were screened using real-time PCR assays. An aliquot of the TSB broth suspension (50 µL) for all TSB-enriched samples was pelleted by centrifugation at 12,000 rcf for 2 minutes, supernatant was discarded, and the pellet was suspended in 100 µL of PCR-grade water. The suspended pellet was lysed at 95 ± 2°C for 5 minutes, then screened using real-time PCR assays.

Vacuum Filter Cassettes

Following spiking, 5 mL of extraction buffer with 0.05% Tween 20 and 30% ethanol (extraction buffer) was added to the conical tube containing the nozzle and tubing and set aside. Six (6) mL total of extraction buffer was used to rinse and recover particulates collected within the VFC by adding 2 mL of extraction buffer in three successive rinse steps. Following the second rinse step, the filter was transferred to the 2 oz. cup containing rinsate. The nozzle and tubing containing 5 mL extraction buffer was sonicated in a sonicating bath for 1 minute, then vortexed for 2 minutes and combined with filter rinsate in the 2 oz. cup. The 2 oz. cup containing filter and 11 mL of extraction buffer were sonicated in a sonicating bath for 3 minutes. As much suspension as possible, typically ~ 8 mL, was transferred to a 15-mL conical tube and the suspension was split in half and used for culture-based microbial analysis described in Section 2.4.4, and RV-PCR analysis as described in Section 2.4.5.

For VFC TSB enrichment, all filters were enriched following spore recovery within the 2 oz. cup by adding 30 mL of TSB, then incubated at 37 ± 2°C for 24 to 48 hours. Turbid TSB broth was then streaked onto three SBA plates for isolation. Colonies with *B. a.* Sterne morphology that were isolated on these streak plates were screened using real-time PCR assays. An aliquot of the TSB broth suspension (50 µL) for all TSB-enriched samples was pelleted by centrifugation at 12,000 rcf for 2 minutes, supernatant was discarded, and the pellet was suspended in 100 µL of PCR-grade water. The suspended pellet was lysed at 95 ± 2°C for 5 minutes, then screened using real-time PCR assays.

2.4.4 Culture Method

Culture-based microbiological analysis was performed on each sample by filtering the recovered extract through MicroFunnel filters (Pall, Cat. 4804) then placing the filters onto solid bacterial growth media and incubating. Serial dilution and spread-plating procedures, as prescribed by the

full EPA *B. anthracis* method (EPA, 2012), were not performed since the spike levels were at/near the detection limit for the assay (i.e., spread-plating 0.1 mL of the undilute extract from a Sponge-Stick spiked with 3,000 spores would have resulted in ~12 CFU if 100% efficient). Accordingly, milliliter volumes of the recovered extract were captured onto MicroFunnel filters in the current study.

Initially, each MicroFunnel filter was pre-wetted with 5 mL of phosphate buffered saline (PBS) with 0.05% Tween (PBST), then 10 mL of PBST was added to each MicroFunnel filter to suspend aliquots, 2-mL and 8-mL for Sponge-Sticks and 1-mL and up to 4-mL for VFCs, of the extract followed by vacuum filtration. The walls of each MicroFunnel filter were rinsed with 10 mL of PBST and filtered through the MicroFunnel filter, then the filter membrane was removed and placed onto SBA media.

For the culture method, colonies with a typical *B. a.* Sterne morphology following overnight incubation at 35 to 37°C were counted to determine percent spore recovery. Typical *B. a.* Sterne morphology on SBA are 2 to 5 mm in diameter, flat or slightly convex with edges that are irregular, have a ground-glass appearance, and are not ß-hemolytic.

For the final four Sponge-Stick trials, the extracted sponge was retained and stored at 2 to 8°C. For samples that did not have *B. a.* Sterne morphology (culture non-detects), 25 mL of TSB was added to the extracted sponge and incubated at 37 ± 2°C for 24 to 48 hours. Following incubation, turbid cultures were streaked for isolation onto SBA plates and examined. If no *B. a.* Sterne colonies were observed, 50 µL of the broth was concentrated and analyzed using real-time PCR assays.

Two different microbiologists enumerated colonies over the course of the project, all of whom were trained by the lead microbiologist on the project to most consistently identify presumptive *B. a.* Sterne based on colony morphology. The lead microbiologist periodically reviewed the enumeration results to help ensure consistency and integrity, which is an important consideration and factor in the application of the method because the culture analysis was subjective to the assessment of colony morphology. There were instances of the presence of presumptive *B. a.* Sterne on samples that should not have any (false positive) and no colonies where there should have been (false negatives). All the results presented for culture analyses are based on presumptive identification of *B. a.* Sterne colonies.

2.4.5 RV-PCR Method

Positive Control Preparation

Genomic deoxyribonucleic acid (DNA) of *B. a.* Sterne was extracted for use as a positive control for RV-PCR based analysis. The *B. a.* Sterne vegetative cell culture that DNA was extracted from originated from the spore stock used for spike/recovery tests. The Wizard® Genomic DNA Kit (Promega, Madison, WI) was used following an internal Battelle method specific for extracting *B. anthracis*. The resulting DNA was quantified by Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, cat. P11496). The purified DNA was assigned a unique lot number, dispensed as multiple aliquots, stored frozen at < -20°C, and used as needed as the positive control for PCR analysis.

Sample Processing (per EPA Method 2017, Second Edition (Shah, 2017))

Following filtration of ~12.5 mL of recovered extract through the Whatman™ Autovial™ filter vials (with polyvinyldiene difluoride [PVDF] membrane; Whatman Cat. AV125NPUAQU), two buffer washes were performed. The first wash was 12.5 mL of cold (4°C) high salt buffer (10X PBS) followed by 12.5 mL of cold (4°C) low salt wash buffer (1X PBS). The top portion of the manifold was then removed and placed into a capping tray with pre-filled luer lock caps to seal the filter vials. Five (5) mL of cold (4°C) Brain Heart Infusion Broth (BHIB) was then added to each filter vial, the vials capped, and then vortex-mixed for 10 minutes on a setting of 7. Images of the manifold and capping tray are depicted in Figure 6. Following the vortex step, the broth was mixed by pipetting up and down ~10 times and a 1-mL aliquot was transferred to a screw cap tube and stored at -20°C as the time zero (T_0) aliquot. The capped filter vials were then incubated overnight (~16 hours) in an incubator shaker set to 37 ± 1°C at 230 rpm.

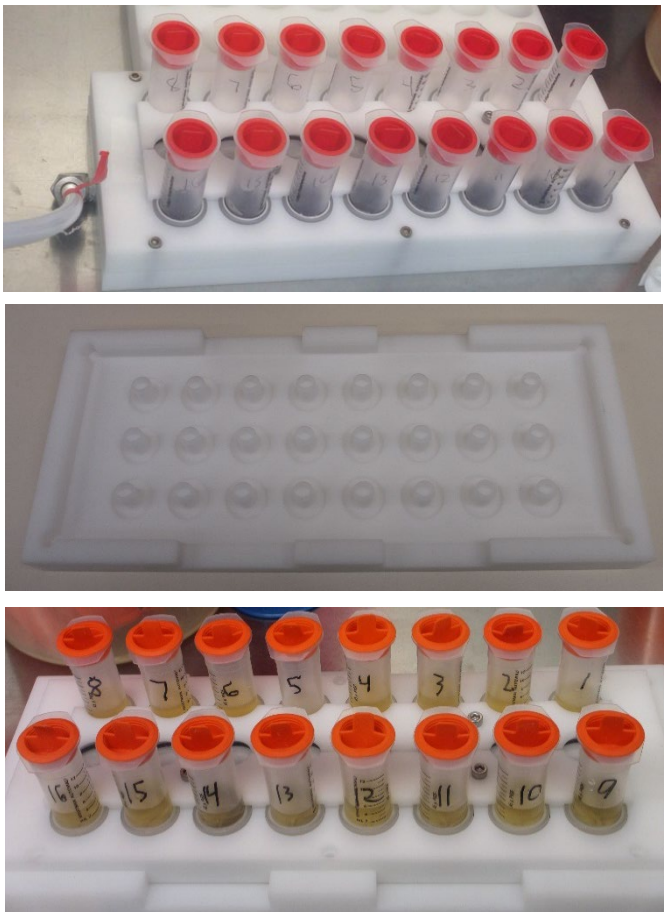

Figure 6. Manifold Containing 16 Filter Vials (Top); Capping Tray (Middle); Capped Filter Vials Containing BHIB (Bottom)

Following overnight incubation (~16 hours) of the filter vials with BHIB, the vials were mixed on the platform vortex for 10 minutes with speed set to 7. (Note, the 16-hour incubation was longer than the 9-hr incubation specified in the "U.S. EPA Protocol for Detection of *Bacillus anthracis* in Environmental Samples During Remediation Phase of an Anthrax Incident" in the 2012 version. The 2017, 2nd Edition of the protocol specified 9 hours, or longer. The 16-hour incubation allowed for a standard work schedule to be maintained rather than require an overnight shift that would have been required by a 9-hour incubation.) The culture suspension was mixed by pipetting up and down ~10 times, and a 1-mL aliquot was transferred to screw cap tubes and labeled as the final time (T_{final}) aliquot.

DNA Extraction and Purification

Prior to extraction of DNA, the lysis buffer with anti-foam reagent and the alcohol wash was added according to the manufacturer's instructions in the Magnesil Blood Genomic, Max Yield

System Kit (Promega, Cat. MD1360) and a heat block was pre-heated to 80°C. All screw-capped, 1-mL aliquots were thawed and centrifuged at 14,000 rpm (18,188 rcf) for 10 minutes (4°C), and 800 µL of the supernatant from each tube was removed and discarded. To extract the DNA, 800 µL of lysis buffer was added to each tube and the samples were mixed by vortexing on high (~1,800 rpm) in 10 second pulses for a total of 60 seconds. Each tube was then vortex-mixed for 10 seconds at low speed directly before the lysate was transferred to a 2-mL labeled Eppendorf tube. The lysate tube was then incubated at room temperature for 5 minutes. Uniformly resuspended paramagnetic particles (PMPs) (600 µL) were added to each lysate tube and the samples were mixed by vortexing. After vortexing each T_0 and T_{final} tube for 10 seconds (high, ~1,800 rpm), the samples were incubated at room temperature for 5 minutes.

The samples were then placed on the magnetic stand with the hinged-side of the tube facing toward the magnet after briefly resuspending the particles by vortexing. The magnetic rack was then inverted to ensure all PMPs were contacting the magnet. After 10 seconds, the tubes were opened, and the liquid removed without disturbing the PMPs. Lysis buffer (360 µL) was then added to each T_0 and T_{final} tube, capped, and vortexed for 10 seconds. The tubes were then placed on the magnetic stand and inverted again. The supernatant was then removed and 360 µL of salt wash solution was added to each tube. The tubes were capped and vortexed for 10 seconds, placed on the magnetic stand, and inverted. The supernatant was removed without disturbing the PMP pellet. The pelleted PMPs were washed a second time with 360 µL of salt wash solution.

After removal of the second salt wash supernatant, 500 µL of alcohol wash was added to each tube. The tubes were vortexed for 10 seconds, placed on the magnetic stand, and inverted. The supernatant was then removed, and two more alcohol washes were conducted for a total of three 500-µL alcohol washes. A fourth alcohol wash was then conducted using 500 µL of 70% ethanol. After the supernatant from the 70% ethanol wash was removed, all tubes were opened and allowed to air dry for 2 minutes. The open tubes were then heated at 80°C in a heat block inside a BSC until the PMPs were dry (~20 minutes). DNA was then eluted from the PMPs by the addition of 200 µL of elution buffer to each T_0 and T_{final} tube. The tubes were then closed, vortexed for 10 seconds, and incubated in the heat block for 80 seconds. The tubes were then vortexed another 10 seconds and incubated in the heating block for a minute. The vortexing and heating for 1 minute was repeated four more times for a total of five times. The tubes were then removed from the heating block and incubated at room temperature for at least 5 minutes. Each

tube was briefly vortexed and then centrifuged at 2,000 rpm (371 rcf) at 4ºC for 1 minute. The tubes were then vortexed and placed on the magnetic stand for at least 30 seconds. The elute was collected (~80 to 90 µL) and transferred to clean, labeled, 1.5-mL tubes on a cold block. The tubes were centrifuged at 14,000 rpm (18,188 rcf) at 4°C for 5 minutes to pellet any particles remaining with the eluted DNA. The supernatant was carefully removed and transferred to a new 1.5-mL tube using a new tip for each tube. The T_0 and T_{final} DNA extracts were stored at 4ºC until RV-PCR analysis or at -20ºC if RV-PCR could not be performed within 24 hours.

RV-PCR Assay

The EPA protocol originally provided (EPA, 2012) uses singleplex, real-time PCR assays for *B. anthracis* detection and quantification. Battelle assessed the feasibility to combine two singleplex assays targeting the chromosome and pXO1 assays described in the EPA protocol into a duplex assay to reduce analysis time and cost associated with filter extract analysis. It was previously demonstrated that the RV-PCR performance was unchanged when conducted using the duplex assay in a single analysis or using the singleplex assays in two independent analyses.

The duplex TaqMan® real-time PCR assay utilized FAM and VIC reporter dyes for detection of two *B. a.* Sterne DNA sequence targets simultaneously in a single reaction. (FAM and VIC are Applied Biosystems trademark fluorescent reporter dyes on 5' end of PCR probe that emit at ~517 nm and ~551 nm, respectively.) The two assays target sequences on the *B. anthracis* chromosome and pXO1 plasmid and were previously described as singleplex real-time PCR assays (Letant et al., 2011). The duplex PCR assay Master Mix was prepared using the conditions provided in Appendix B. Each sample DNA extract was assayed in triplicate reactions. Controls consisted of four positive control wells containing 50 picogram (pg) of DNA extracted from *B. a.* Sterne 34F2 (NR-1400, BEI Resources) and four no template controls (NTCs) were also included with each assay. Applied Biosystems 7500 Fast Real-Time PCR Instrument was used for PCR assay development and testing. Thermocycler conditions with a fast ramp rate were:

- Stage 1: 1 cycle at 95°C for 20 sec
- Stage 2: 45 cycles at 95°C for 3 sec followed by 60°C for 30 sec

Note, the Stage 1 cycle conditions were slightly modified from the original EPA method and concurrence to proceed with the revisions was provided by EPA (Shah, 2018).

2.5 Method Implementation

The primary microbial methods used to spike/recover/analyze the Sponge-Sticks and VFCs, shown as they occur in chronological order, are depicted graphically in the process flow diagram of Figure 7.

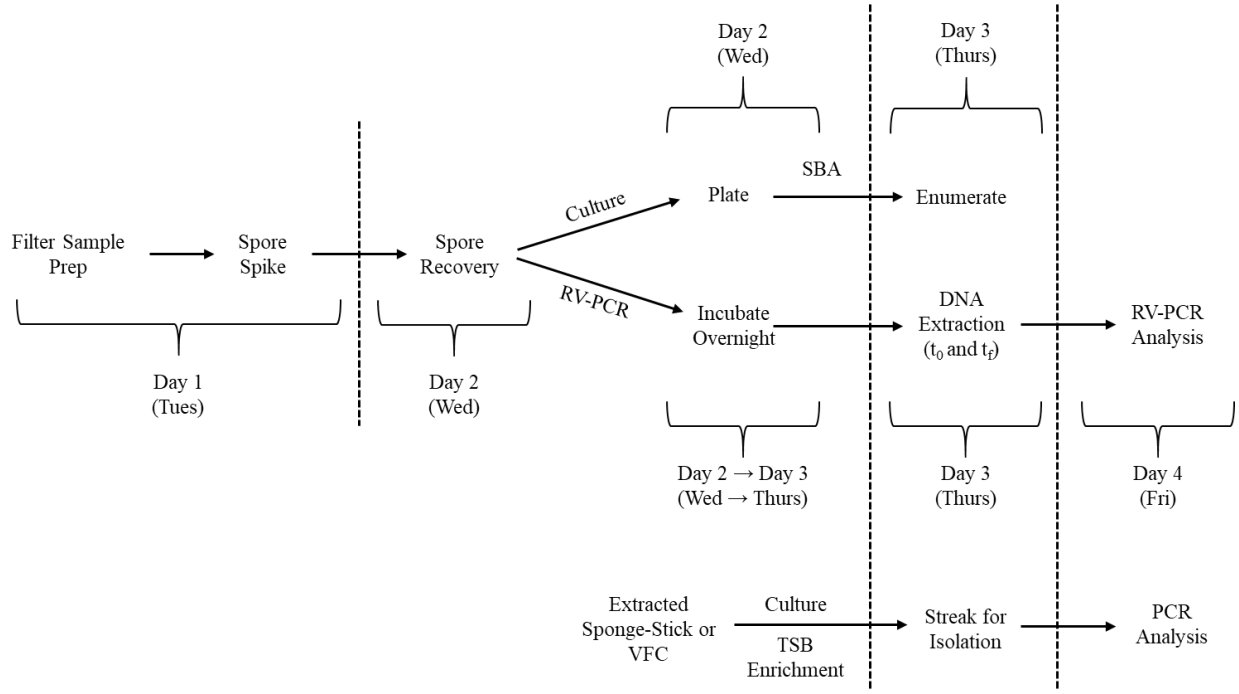

Figure 7. Process Flow Chart Depicting Key Method Process Steps in Chronological Order

The method implemented, in the form of work instructions followed by the analytical staff, is provided in Appendices C through N. These work instructions also complement those microbiological methods described in Section 2.4, and emphasize glove-changing schedules that were implemented to minimize cross-contamination. The work instructions were reviewed in detail during an in-progress review (Calfee, 2018).

The above method was used to analyze a batch of 16 filter samples per trial, with 1 trial conducted per week. For each weekly test, Sponge-Sticks or VFCs were spiked using *B. a.* Sterne spores suspended in water (Sponge-Sticks) or 50% ethanol (VFCs). Each test consisted of samples loaded with 0, 30, 300 or 3,000 spores per "Work Instruction for Spiking with *Bacillus anthracis* Spores – Sponge-Sticks or VFC" in Appendix C or D, respectively. The spiked filters were recovered following the "Work Instruction for *Bacillus anthracis* Spore Recovery – Sponge-Sticks or VFC," as detailed in Appendix E or F, respectively. The recovered

volume was then split between the traditional culture method and RV-PCR. The culture aliquot was divided into 2-mL or 8-mL aliquots for Sponge-Sticks and 1-mL or ≤ 3-mL aliquots (remaining volume without transferring settled particulates) for VFCs and plated onto media and incubated overnight as outlined in the "Work Instruction for Culture of *Bacillus anthracis* Spores Recovered – Sponge-Sticks or VFC" in Appendix G or H, respectively. The T_0 RV-PCR aliquot was stored frozen while the recovered spores enrich overnight, then the T_{final} aliquot was removed and the DNA was extracted from both T_0 and T_{final} aliquots per "Work Instruction for Manual DNA Extraction and Purification from *Bacillus anthracis"* in Appendix I. The extracted DNA was then analyzed using a duplex real-time PCR assay targeting the chromosome and pXO1 of *B. anthracis* per "Work Instruction for RV-PCR for *Bacillus anthracis* Spores – Sponge-Sticks or VFC" in Appendix J or K, respectively. PCR was also used to confirm or refute presumptive *B. a.* Sterne spores selected from the culture analysis per "Work Instruction for Selecting Presumptive *B. a.* Sterne Colonies for qPCR Confirmation" in Appendix L. Selected samples for which the culture was a non-detect were further analyzed using an enrichment procedure per "Work Instruction for TSB Enrichment for Culture non-Detects – Sponge-Sticks or VFC," in Appendix M or N, respectively.

2.6 Data Reduction and Analysis

2.6.1 Culture – Percent Recovery

The percent recovery efficiency ($E_{recovery}$) of *B. a.* Sterne from each spiked surface sample was calculated by dividing the number of presumptive *B. a.* Sterne CFUs recovered ($N_{recover}$) from the filter by the actual number of *B. a.* Sterne spores spiked (N_{spike}) onto the filter (determined from the stock suspension titer for each test), then multiplied by 100. $N_{recover}$ is a product of the presumptive *B. a.* Sterne spore concentration ($C_{recover}$) (CFU/mL) and the total volume of extract used to recover the spores ($V_{extract}$) (mL). Mathematically, the percent recovery is expressed as follows:

$$E_{recovery}(\%) = \frac{C_{recover} * V_{extract}}{N_{spike}} * 100\%$$

Further, the number of presumptive *B. a.* Sterne spores present in the volume of extract collected onto the MicroFunnel filter membrane was divided by the extract volume analyzed; 2 mL and 8 mL for Sponge-Sticks or 1 mL and ≤ 3 mL for VFCs to yield a presumptive *B. a.* Sterne spore concentration ($C_{recover}$) (CFU/mL). The extract volume ($V_{extract}$) (~25 mL for Sponge-Sticks and 11 mL for VFCs) were used to determine *B. a.* Sterne CFUs recovered from the filter sample. The percent recovery was calculated for both the low-volume (2 mL or 1 mL for Sponge-Sticks and VFCs, respectively) and high-volume (8 mL or ≤ 3 mL for Sponge-Sticks and VFCs, respectively) aliquots. The reported percent recovery was determined using the below rules:

1) Report the percent recovery from the aliquot (low-volume or high-volume) that has between 20 to 80 CFU.

2) Report the high-volume aliquot percent recovery if the CFU counted from both aliquots is less than 20.

3) Report the high-volume aliquot percent recovery if the CFU counted from both aliquots is between 20 to 80.

4) Report the low-volume aliquot percent recovery if the background flora on the high-volume aliquot produces numerous colonies or a lawn of growth, thus complicating the identification of *B. a.* Sterne colonies.

The number of CFUs are reported as presumptive *B. a.* Sterne colonies. PCR analysis of presumptive colonies was required to positively confirm the presence of *B. a.* Sterne. To perform this task, a portion (~10 CFUs per trial) of the presumptive colonies was collected into 100 µL of PCR-grade water in microcentrifuge tubes. The colony suspension was then heated for 5 minutes on a heat block at 95°C. The lysate was cooled and then centrifuged at 14,000 rpm (18,188 rcf) for 2 minutes and the supernatant was analyzed using the real-time PCR assays targeting the *B. anthracis* chromosome and pXO1 gene targets.

2.6.2 RV-PCR

The cycle threshold (Ct) values for the T_0 and T_{final} timepoints as well as the delta Ct value (ΔCt) were reported. The ΔCt is generated by subtracting the average Ct (from triplicate reactions) generated by the T_{final} aliquot from the average Ct (triplicate reactions) value generated by the T_0 aliquot. A positive ΔCt value indicates that viable *B. a.* Sterne spores were detected in the sample if all the below acceptance criteria were met:

- The ΔCt must be greater than or equal to 9 for both the chromosome and pXO1 targets ($\Delta Ct = Ct(T_0) - Ct(T_{final}) \geq 9$)

Additional criteria exist for the positive confirmation of a sample if analyzing samples obtained from an actual incident, but for this study the above criterion was used (i.e., three PCR targets (chromosome, pX01, and pX02) are utilized for RV-PCR analysis of fully virulent *Bacillus anthracis*).

2.6.3 Presentation of Results

The method employed to recover *B. a.* Sterne spores spiked onto the samples was consistent with current EPA methods, as described in Section 2.4.4. In the instance of an actual biological release, the entire extract would be analyzed either using a culture method or a RV-PCR method (Calfee, 2018). In the study performed and reported here, however, the sample extract was split as described in Section 2.4.4 and 2.4.5, so that approximately half of the sample extract was used for culture analysis and the other half for RV-PCR analysis. Consequently, neither the culture nor the RV-PCR had the potential maximum quantity (assuming 100% recovery efficiency from the filter) of spores available in the extract for analysis. Rather, each split sample extract had a maximum of nominally half the actual spiked spore quantity available for their respective analyses. Therefore, in the presentation of results in tables and figures, unless explicitly noted otherwise, column headers or axes labels denote the nominal maximum number of spores available in the sample for its respective analysis, which was half of the target spore load.

For example, results were presented in plots of both spore recovery efficiency for the culture analyses and of ΔCt for RV-PCR analyses with an x-axis title of "Nominal Spores Available for Analysis (CFU)" with an x-axis label of 0, 15, 150, and 1,500. This convention of presenting the results was considered the most accurate and consistent representation and allowed for the most unambiguous discussion and interpretation of results across all the filter types and analytical methods, recognizing that the filters were originally spiked with target quantities of 0, 30, 300, and 3,000 *B. a.* Sterne spores.

As described in Section 2.4.2, the samples were spiked with a target quantity of spores by applying twenty (20) 5-µL drops of a *B. a.* Sterne spore stock suspension with a target titer of 30,000 spores/mL, diluted in log increments. The reported spore load for each filter analyzed was based on the *B. a.* Sterne spore suspension titer measured for each test trial in CFU. As expected, there was variability in the measured spore titer for each trial. Consequently, the summary tables of results also contain the average (± one standard deviation) of the measured or determined quantity of spores spiked onto the filter, which provides the reader with information other than the nominal spore load as defined in the test matrices to aide with interpretation of the results.

3.0 RESULTS AND DISCUSSION

As described in the previous section, all results presented in plots have an x-axis title and labels of 0, 15, 150, and 1,500 CFU representing the nominal spores available for analysis. Similarly, the summary results in the tables contain the same nominal quantity of spores available, and the determined quantity of spores applied to the surface sampler substrate being assessed. This convention of presenting the results was considered the most accurate and consistent representation and allowed for the most unambiguous discussion and interpretation of results across all the filter types and analytical methods, recognizing that the filters were originally spiked with target quantities of *B. a.* Sterne spores of 0, 30, 300, and 3,000 but extract samples were split in approximately equal volume for the two analyses.

Note, the spores available for analysis represent the maximum number of spores available (assumes 100% recovery from the filter and no physical losses associated with processing of samples); it is not an absolute indication of the analytical method's limit of identification. Rather, it is a measure of the method's end-to-end performance to identify *B. a.* Sterne.

3.1 Sponge-Stick Analyses Results

3.1.1 Culture Method

A summary of the average and standard deviation of the measured recovery efficiencies of presumptive *B. a.* Sterne spores recovered from the Sponge-Stick samples spiked with *B. a.* Sterne and using SBA medium are presented in Table 4. The determined number of spores available and the number of presumptive *B. a.* Sterne spores recovered are tabulated along with the nominal quantity of spores available for analysis (15, 150 and 1,500 CFU/filter sample). The presumptive *B. a.* Sterne recovery efficiencies on the SBA plates are plotted in Figure 8 through Figure 25, one plot for each surface sampled with a Sponge-Stick. (There is no plot for the Street Grate surface since the recovery efficiency was 0% for all nominal spores available conditions.) Note, a percent recovery is not tabulated nor plotted for the 0-spore spike condition since, by definition, a meaningful recovery efficiency cannot be calculated, even though there could have been presumptive *B. a.* Sterne colonies counted based on colony morphology. However, any presumptive *B. a.* Sterne CFUs for the 0-spike condition were reported in Table 4. The quantity of presumptive *B. a.* Sterne colonies for each Sponge-Stick sample used in the percent recovery calculations are reported in Appendix O.

Table 4. Recovery Efficiencies for Presumptive *B. a.* Sterne Spores from Sponge-Stick Surface Samples Cultured in SBA Medium

Surface Sample (Surface ID)	Sample Reps	Spores Available for Analysis (CFU)		Spore Recovery (CFU) (X ± σ)[c]	Spore Recovery Efficiency (%) (X ± σ)[d]
		Nominal[a]	Determined (X ± σ) [b]		
Floor Tile (FLTILE)	3	0	0	0	N/A
	5	15	14 ± 2.8	8.7 ± 13	61 ± 83
	5	150	140 ± 28	16 ± 20	13 ± 18
	5	1,500	1,400 ± 280	300 ± 120	22 ± 7.5
Floor (Concrete) (FLCON)	3	0	0	0	N/A
	5	15	14 ± 2.5	6.2 ± 7.7	44 ± 50
	5	150	140 ± 25	23 ± 9.6	18 ± 9.4
	5	1,500	1,400 ± 250	320 ± 73	24 ± 5.6
Step (Metal) (STEPS)	3	0	0	0	N/A
	5	15	12 ± 7.0	18 ± 41	110 ± 240
	5	150	140 ± 40	9.9 ± 22	8.3 ± 19
	5	1,500	1,500 ± 440	200 ± 92	15 ± 11
Wall Tile (WLTILE)	3	0	0	0	N/A
	5	15	14 ± 4.2	5.2 ± 4.2	37 ± 31
	5	150	140 ± 42	33 ± 21	23 ± 12
	5	1,500	1,400 ± 420	290 ± 170	23 ± 16
Glass Window (GLSWIN)	3	0	0	0	N/A
	5	15	16 ± 3.9	5.9 ± 3.9	36 ± 18
	5	150	160 ± 41	45 ± 17	27 ± 5.5
	5	1,500	1,600 ± 410	320 ± 30	21 ± 6.4
Electronic Display Panel (Below Ground) (EDPAN(B))	3	0	0	0	N/A
	5	15	14 ± 7.3	4.8 ± 4.9	28 ± 29
	5	150	160 ± 36	35 ± 12	22 ± 4.8
	5	1,500	1,600 ± 360	350 ± 50	24 ± 11
Glass Panel (GLSPAN)	3	0	0	0	N/A
	5	15	20 ± 5.9	7.8 ± 5.2	41 ± 25
	5	150	190 ± 55	60 ± 16	33 ± 10
	5	1,500	1,900 ± 550	510 ± 110	28 ± 7.1
Fluorescent Light Fixture (FLLFIX)	3	0	0	0	N/A
	5	15	20 ± 5.3	5.8 ± 4.6	30 ± 30
	5	150	180 ± 56	42 ± 13	24 ± 5.6
	5	1,500	1,800 ± 560	430 ± 96	25 ± 3.5
Overhead Sign (OHSIGN)	3	0	0	0	N/A
	5	15	10 ± 2.6	3.1 ± 1.1	32 ± 15
	5	150	100 ± 26	33 ± 14	31 ± 7.2
	5	1,500	1,000 ± 260	320 ± 60	33 ± 12
Metro Card Machine (MCMACH)	3	0	0	0	N/A
	5	15	91 ± 1.6	1.6 ± 2.7	19 ± 31
	5	150	100 ± 23	14 ± 18	20 ± 19
	5	1,500	1,000 ± 230	270 ± 200	26 ± 18

Table 4. Recovery Efficiencies for Presumptive *B. a.* Sterne Spores from Sponge-Stick Surface Samples Cultured in SBA Medium (Cont.)

Surface Sample (Surface ID)	Sample Reps	Spores Available for Analysis (CFU)		Spore Recovery (CFU) (X ± σ)[c]	Spore Recovery Efficiency (%) (X ± σ)[d]
		Nominal[a]	Determined (X ± σ) [b]		
Subway Car Filter Grille (SCGRIL)	3	0	0	0	N/A
	5	15	15 ± 3.5	1.3 ± 2.9	7.7 ± 17
	5	150	140 ± 34	26 ± 17	17 ± 10
	5	1,500	1,500 ± 350	330 ± 130	21 ± 5.5
Control Field Blank (FLDBLK)	3	0	0	0	N/A
	5	15	12 ± 3.0	6.2 ± 3.2	51 ± 23
	5	150	130 ± 27	52 ± 6.3	41 ± 9.5
	5	1,500	1,300 ± 230	460 ± 55	36 ± 9.6
Sidewalk (Concrete) (SWCON)	3	0	0	0	N/A
	5	15	15 ± 2.7	3.6 ± 3.3	25 ± 24
	5	150	150 ± 27	30 ± 15	21 ± 12
	5	1,500	1,500 ± 270	200 ± 84	14 ± 8.7
Electronic Display Pane (Above Ground) (EDPAN(A))	3	0	0	0	N/A
	5	15	15 ± 3.2	4.0 ± 4.9	30 ± 33
	5	150	150 ± 32	49 ± 16	34 ± 8.1
	5	1,500	1,500 ± 320	340 ± 140	24 ± 8.8
Crosswalk Signal (CWSIGN)	3	0	0	0	N/A
	5	15	15 ± 3.9	2.4 ± 5.4	13 ± 29
	5	150	150 ± 43	22 ± 20	13 ± 10
	5	1,500	1,500 ± 430	300 ± 53	21 ± 6.7
Telephone Booth (TELEBO)	3	0	0	15 ± 25	N/A
	5	15	17 ± 1.8	5.6 ± 11	36 ± 70
	5	150	170 ± 18	28 ± 23	16 ± 13
	5	1,500	1,700 ± 180	300 ± 140	18 ± 7.5
Street Grate (STGRAT)	3	0	0	0	N/A
	5	15	12 ± 6.3	0.0 ± 0.0	0.0 ± 0.0
	5	150	140 ± 28	0.0 ± 0.0	0.0 ± 0.0
	5	1,500	1,400 ± 280	0.0 ± 0.0	0.0 ± 0.0
Crosswalk (Painted) (CWPNTD)	3	0	0	0	N/A
	5	15	8.8 ± 4.5	0.0 ± 0.0	0.0 ± 0.0
	5	150	110 ± 12	4.9 ± 8.0	4.4 ± 6.7
	5	1,500	1,100 ± 120	91 ± 91	8.3 ± 8.0
Granite Bench (GRNBEN)	3	0	0	0	N/A
	5	15	11 ± 2.7	1.5 ± 2.7	13 ± 25
	5	150	130 ± 42	28 ± 11	22 ± 6.2
	5	1,500	1,300 ± 420	220 ± 80	19 ± 12

(a) Nominally one-half of the target spore load on the filter and assuming 100% recovery of spores.

(b) Based on the spiking suspension titer measured each test trial, 100% recovery efficiency, and one-half of extract used for culture analysis.

(c) Presumptive *B. a.* Sterne colonies based on morphology, and one-half of extract used for culture analysis.

(d) Calculated using the actual spore loading on each filter and total presumptive *B. a.* Sterne spores recovered on each Sponge-Stick sample.

The percent recovery efficiency for presumptive *B. a.* Sterne spores generally range between 10 to 50% for most surface samples for all nominal spores available condition (15, 150, and 1,500) with no consistent trend related to the quantity of spores available for analysis. The percent recoveries were within a range of 15 to 40% for the 150 and 1,500 nominal spores available condition, which includes the field blank samples that had an average percent recovery of 52, 40, and 36% for a nominal *B. a.* Sterne spore load of 15, 150, and 1,500, respectively. The Street Grate and Painted Crosswalk samples were the only exception to this result. The Street Grate samples, regardless of the nominal spore load, had no identifiable presumptive *B. a.* Sterne recovered. The Painted Crosswalk had no identifiable presumptive *B. a.* Sterne recovered at 15 nominal spore load and very few at the 150 and 1,500 spores available condition, resulting in recovery efficiencies < 10%. In some instances, the culture plates had organism growth (not of a *B. a.* Sterne morphology) resulting in > 100 colonies that was likely masking colonies of a *B. a.* Sterne morphology. When no presumptive *B. a.* Sterne spores were identified from spike samples, the result was noted as a false negative.

Sponge-Stick sample recovery efficiencies associated with a nominal 15 *B. a.* Sterne spores available generally had a higher standard deviation than those samples with the 150 and 1,500 nominal spores available condition, which was attributed to the relatively few (< 10) recovered presumptive *B. a.* Sterne colonies. For the Steps (Metal) surface sample the average recovery efficiency exceeded 100%, which is clearly an over-estimation of recovered spores. The infeasibly high spore recovery efficiencies (> 100%) was attributed to presence of background flora on those filters with a colony morphology that was indistinguishable from *B. a.* Sterne, and thus counted as a presumptive *B. a.* Sterne spore. On average, with 100% recovery, the 8-mL aliquot plated would have 5 spores to enumerate. Samples with the 150 and 1,500 spore condition generally have a lower standard deviation compared to the 15-spore condition because more actual *B. a.* Sterne spores are available to enumerate. As discussed in Section 2.4.4, the method for determining the number of *B. a.* Sterne spores recovered was determined based on colony morphology, and thus susceptible to biasing high due to non-*B. a.* Sterne organisms exhibiting an indistinguishable morphology to the microbiologist counting the colonies.

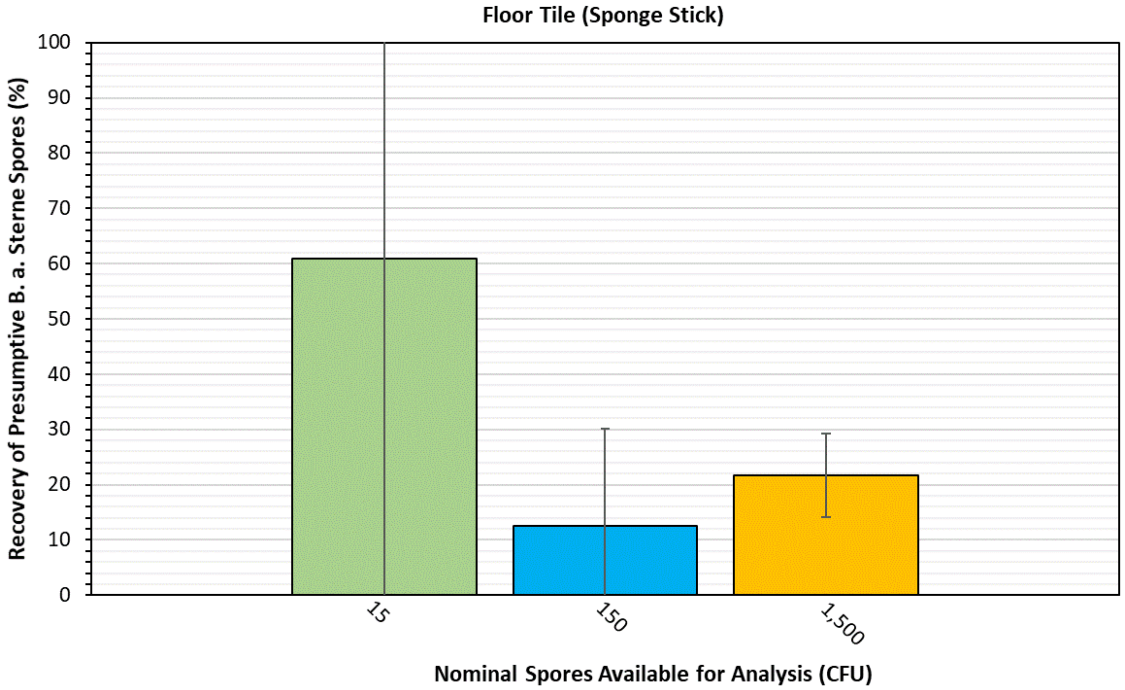

Figure 8. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Floor Tile Sponge-Stick Samples Using SBA Medium

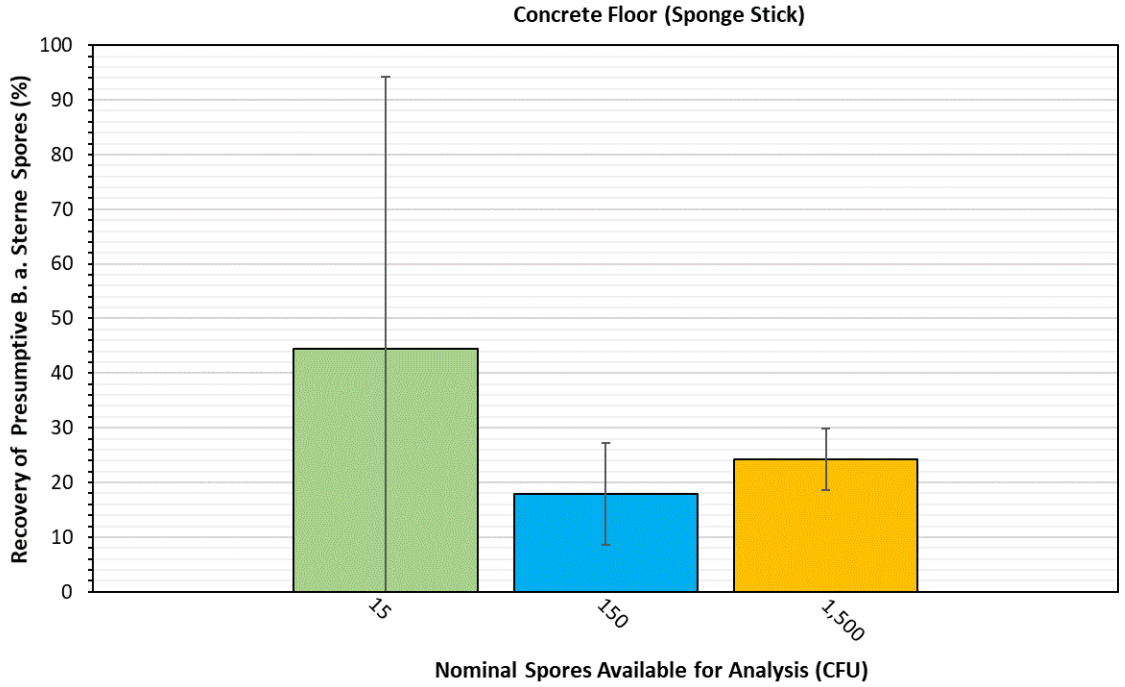

Figure 9. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Concrete Floor Sponge-Stick Samples Using SBA Medium

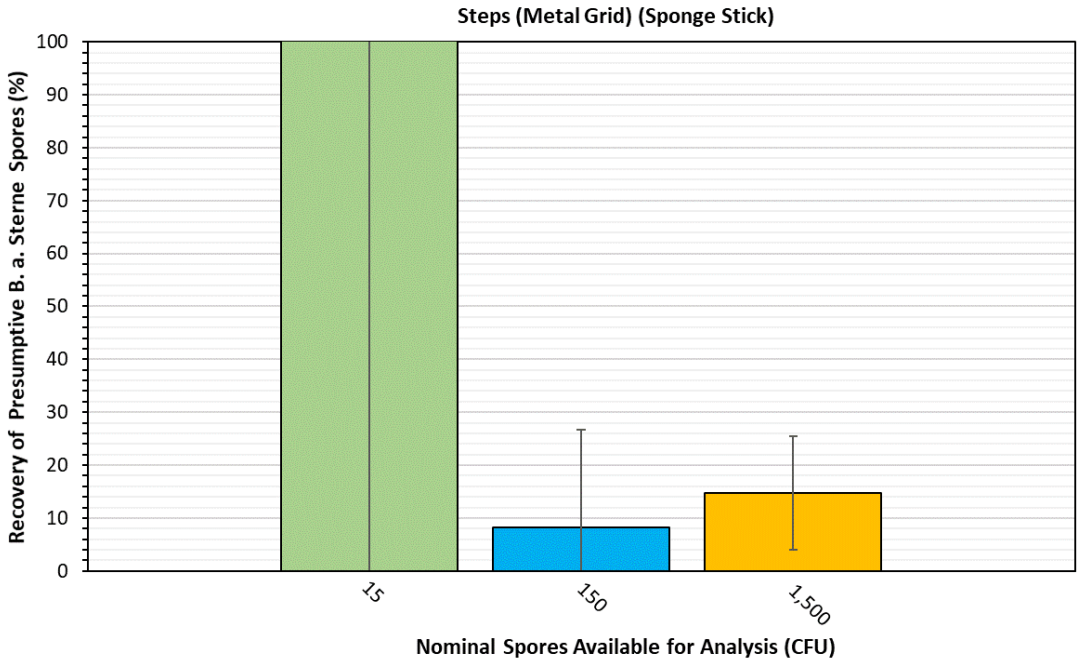

Figure 10. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Steps (Metal) Sponge-Stick Samples Using SBA Medium

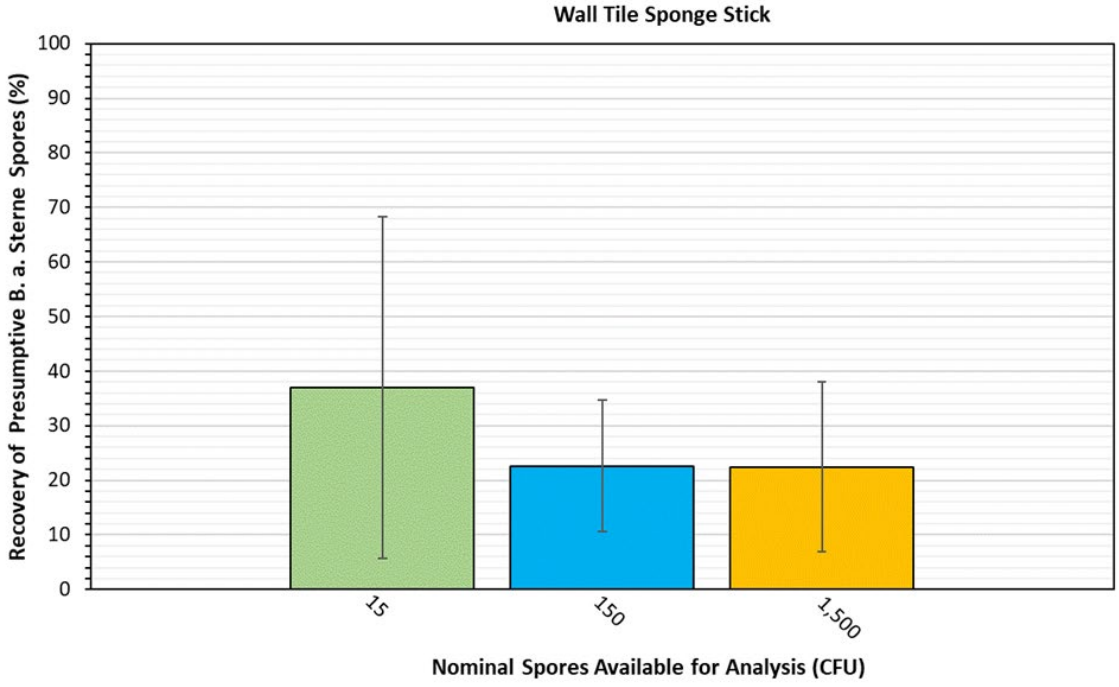

Figure 11. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Wall Tile Sponge-Stick Samples Using SBA Medium

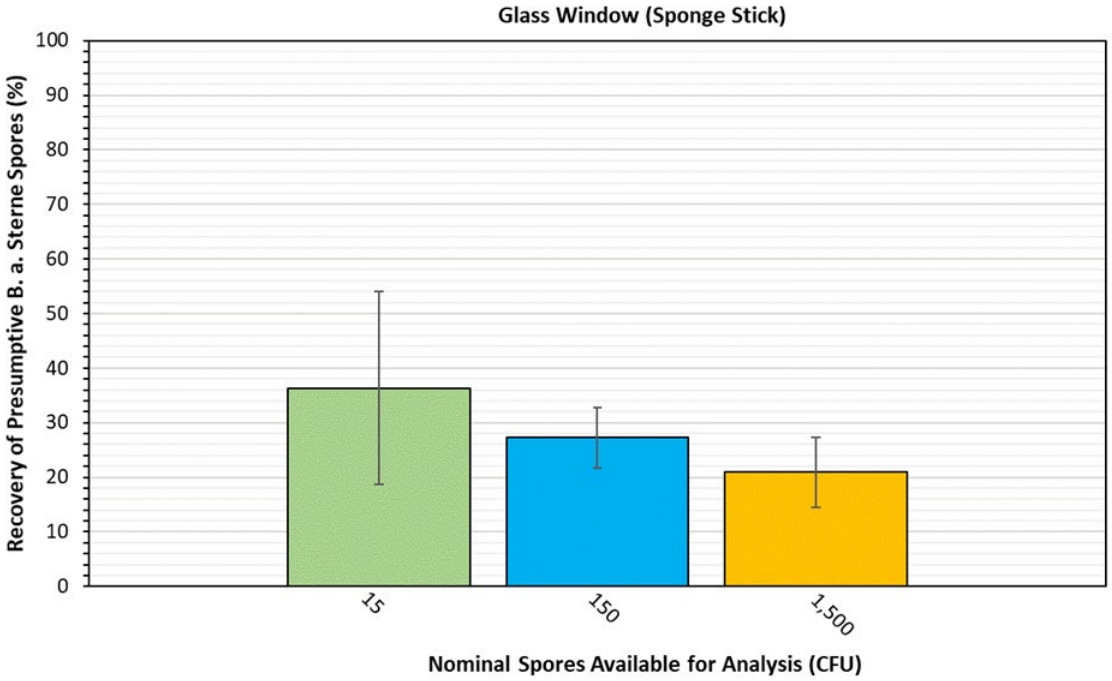

Figure 12. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Glass Window Sponge-Stick Samples Using SBA Medium

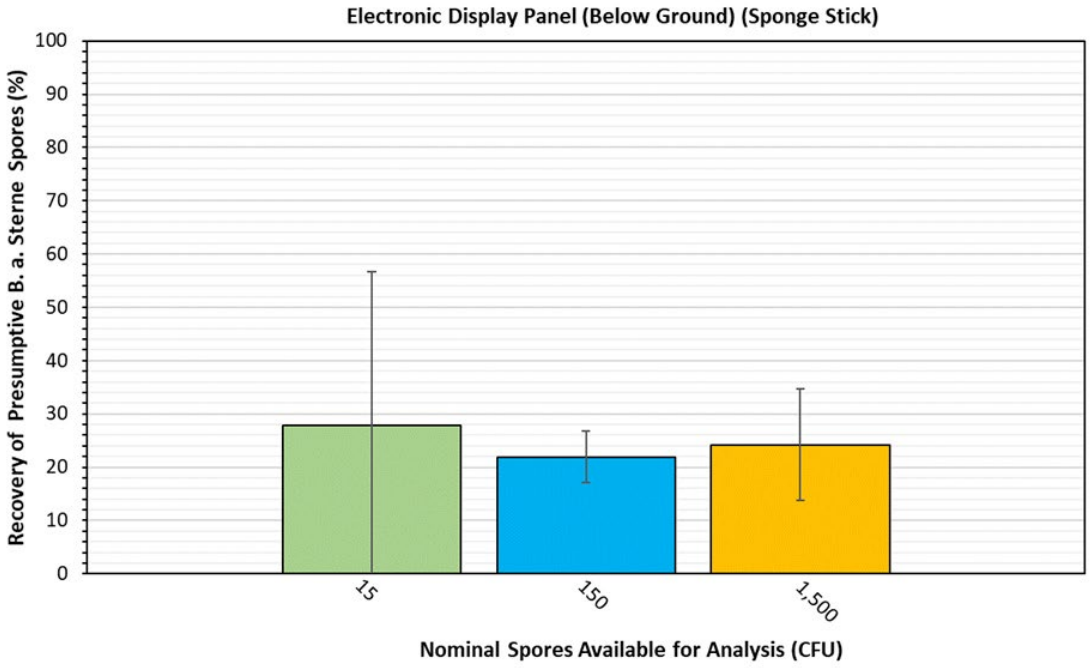

Figure 13. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Electronic Display Panel (Below Ground) Sponge-Stick Samples Using SBA Medium

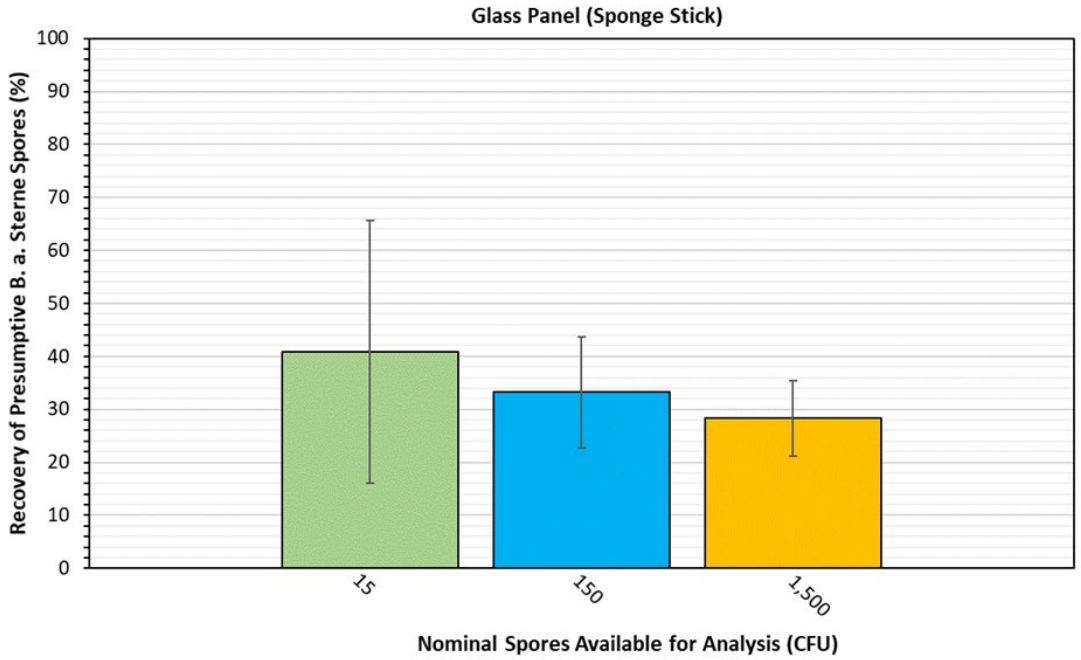

Figure 14. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Glass Panel Sponge-Stick Samples Using SBA Medium

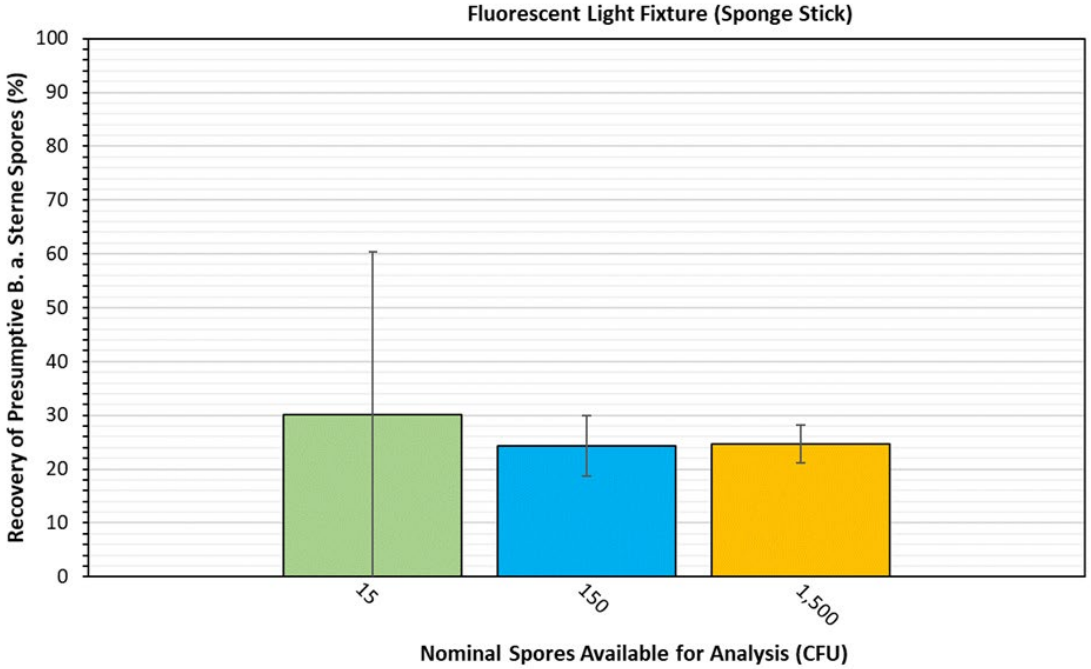

Figure 15. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Fluorescent Light Fixture Sponge-Stick Samples Using SBA Medium

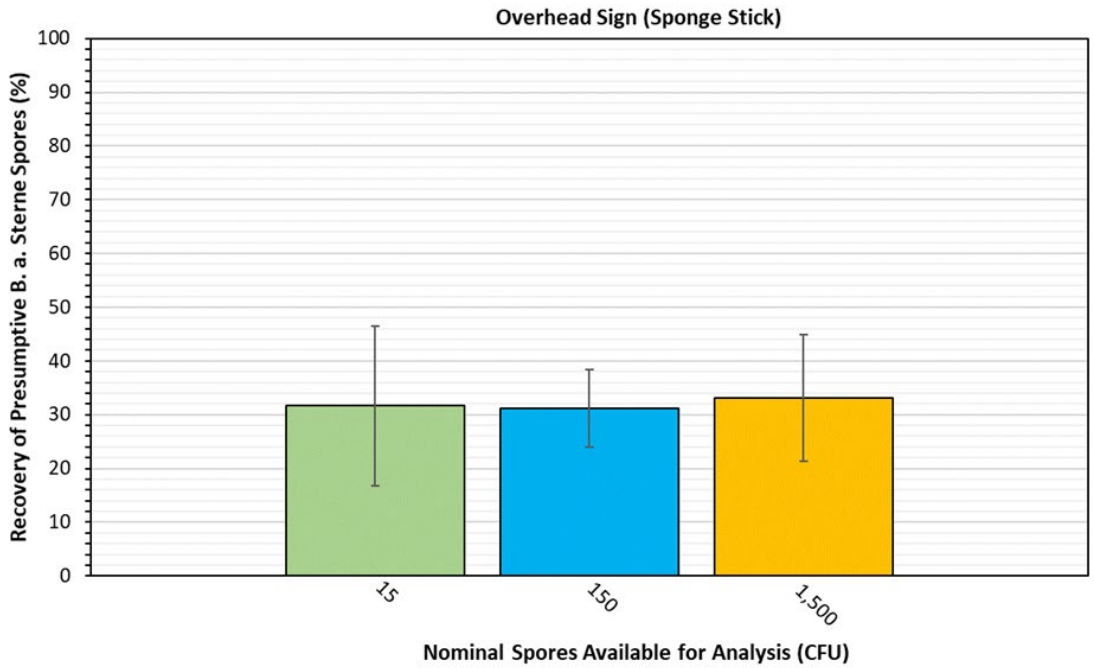

Figure 16. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Overhead Sign Sponge-Stick Samples Using SBA Medium

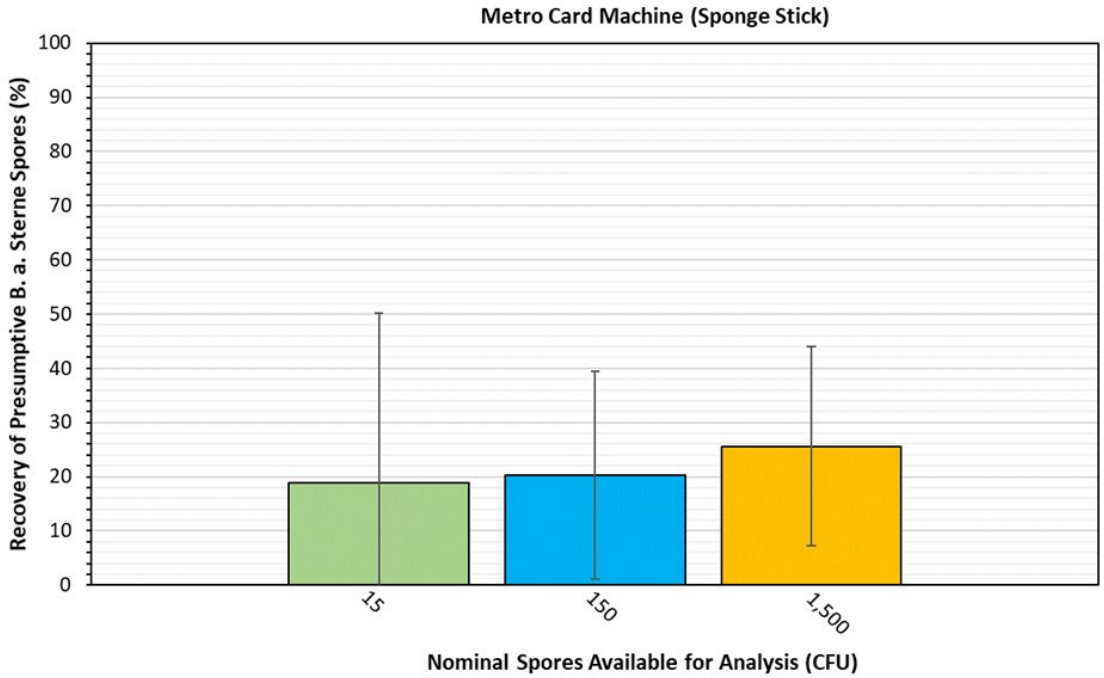

Figure 17. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Metro Card Machine Sponge-Stick Samples Using SBA Medium

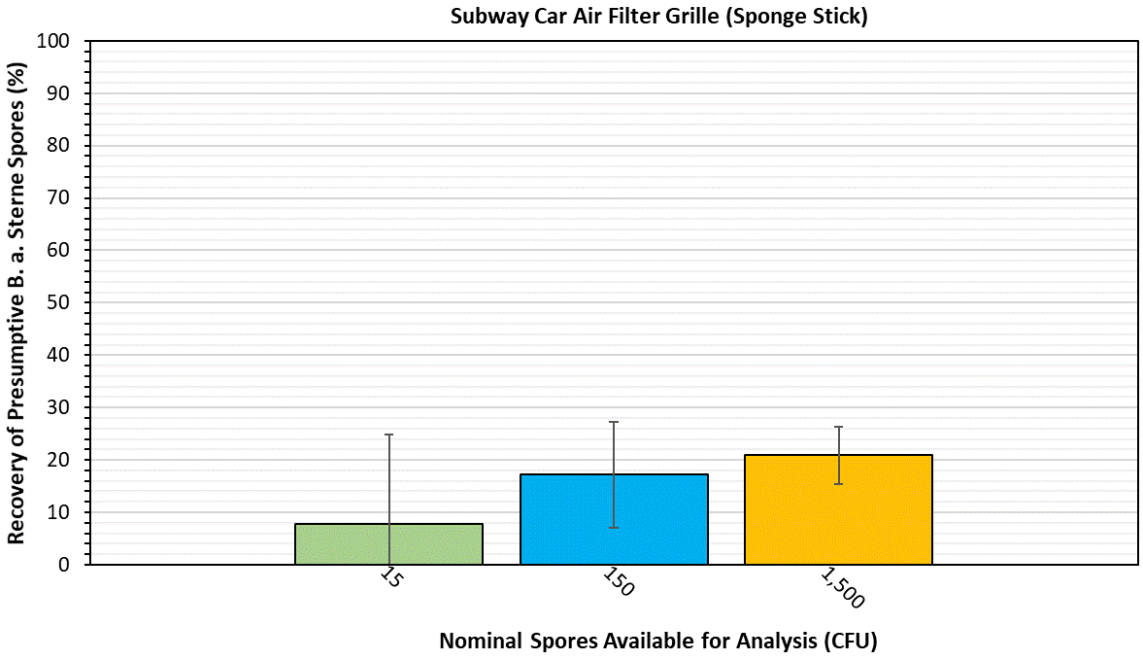

Figure 18. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Subway Car Filter Grille Sponge-Stick Samples Using SBA Medium

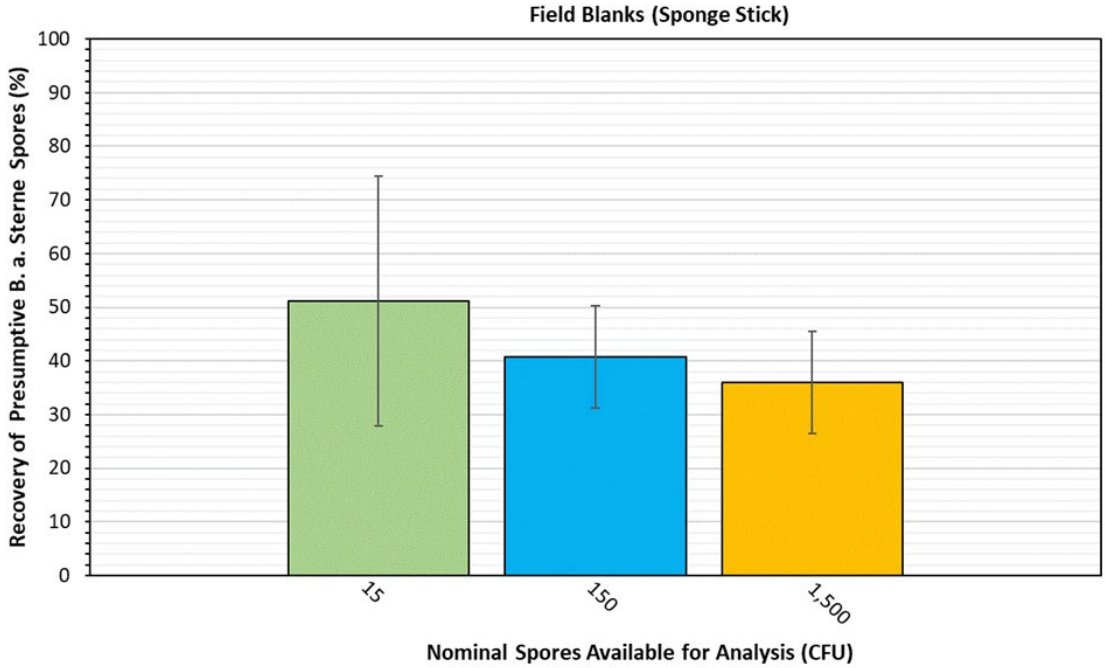

Figure 19. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Field Blank Sponge-Stick Samples Using SBA Medium

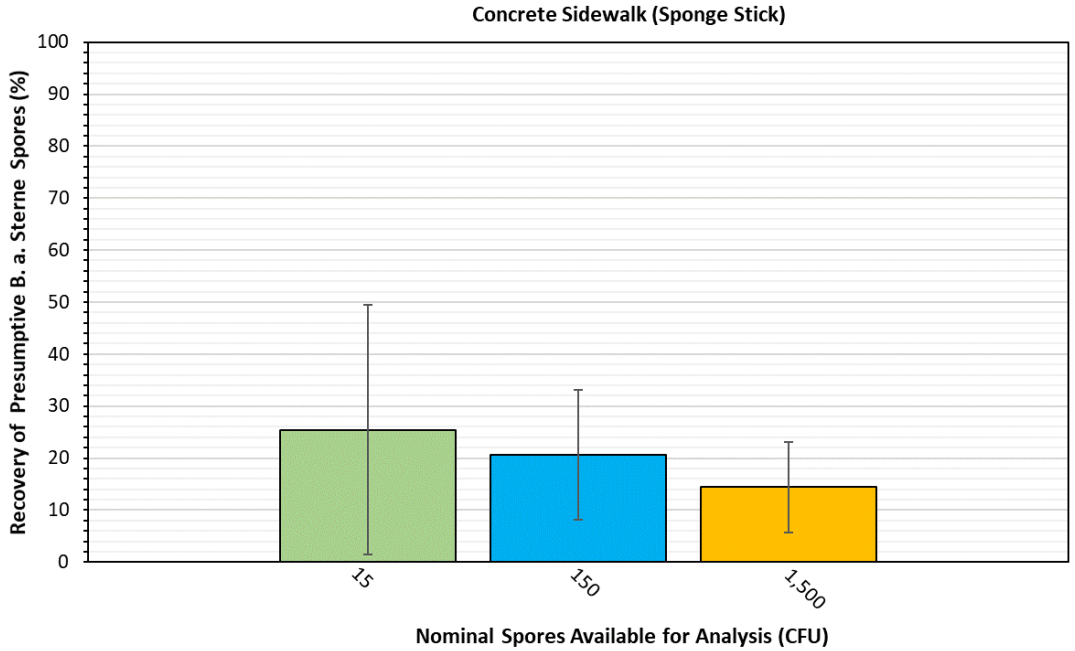

Figure 20. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Concrete Sidewalk Sponge-Stick Samples Using SBA Medium

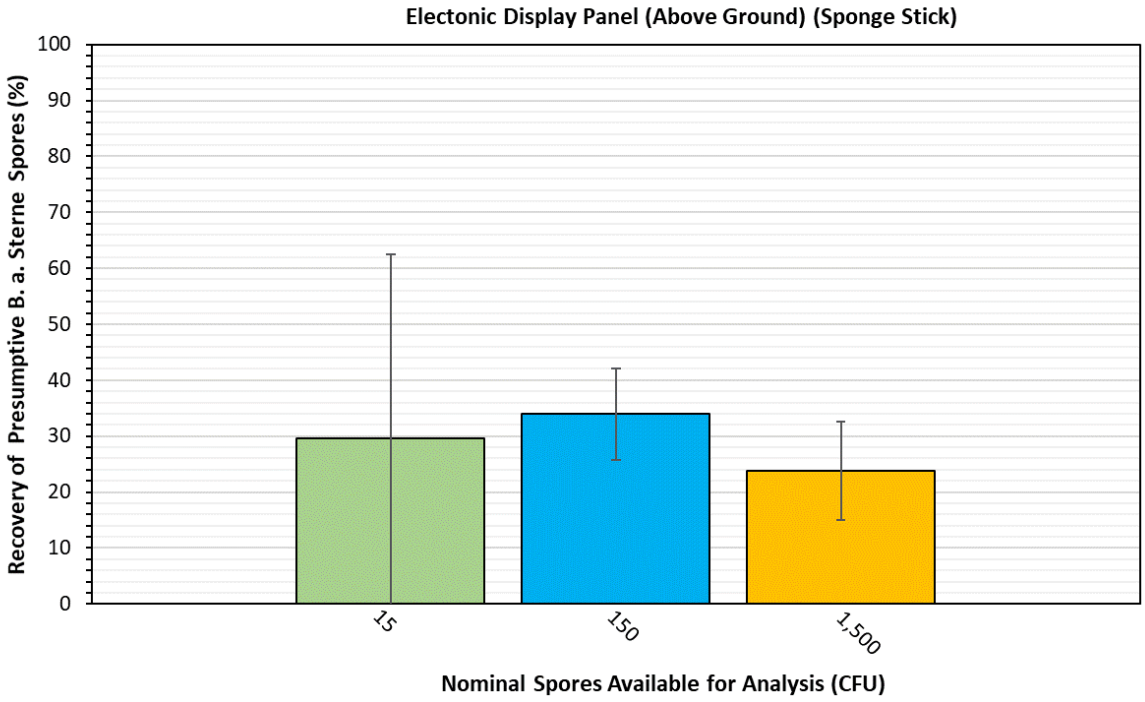

Figure 21. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Electronic Display Panel (Above Ground) Sponge-Stick Samples Using SBA Medium

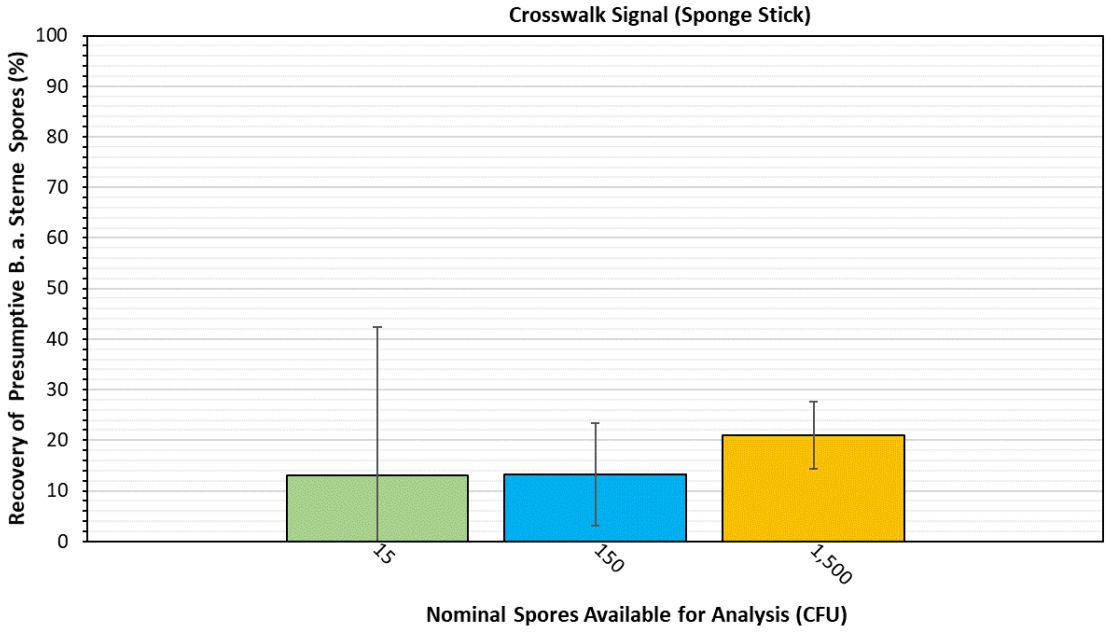

Figure 22. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Crosswalk Signal Sponge-Stick Samples Using SBA Medium

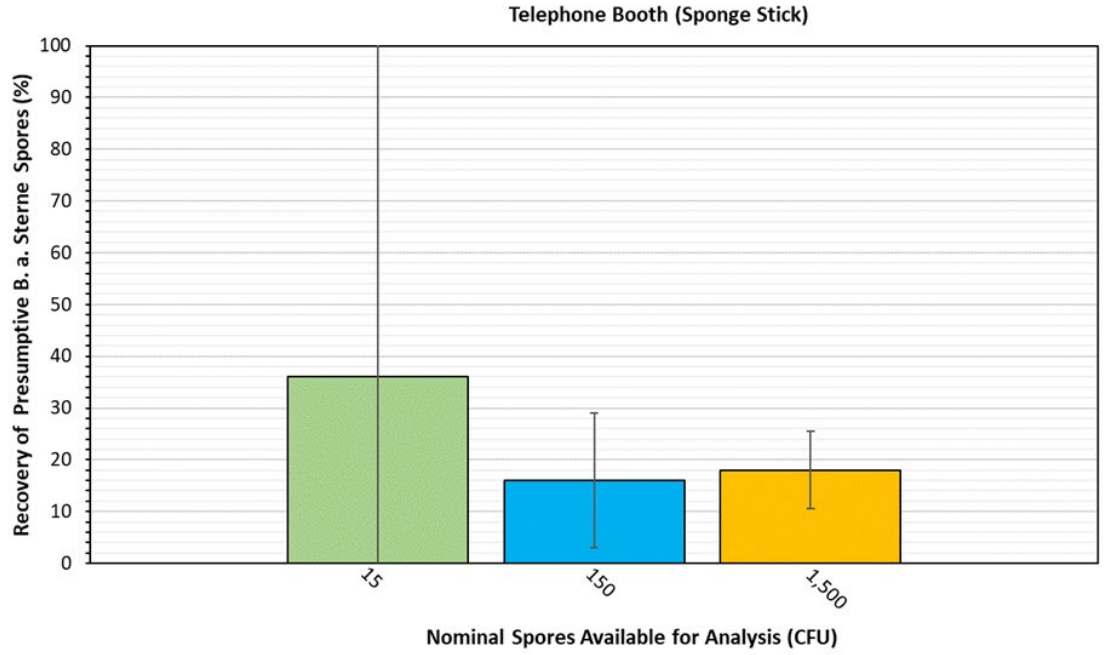

Figure 23. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Telephone Booth Sponge-Stick Samples Using SBA Medium

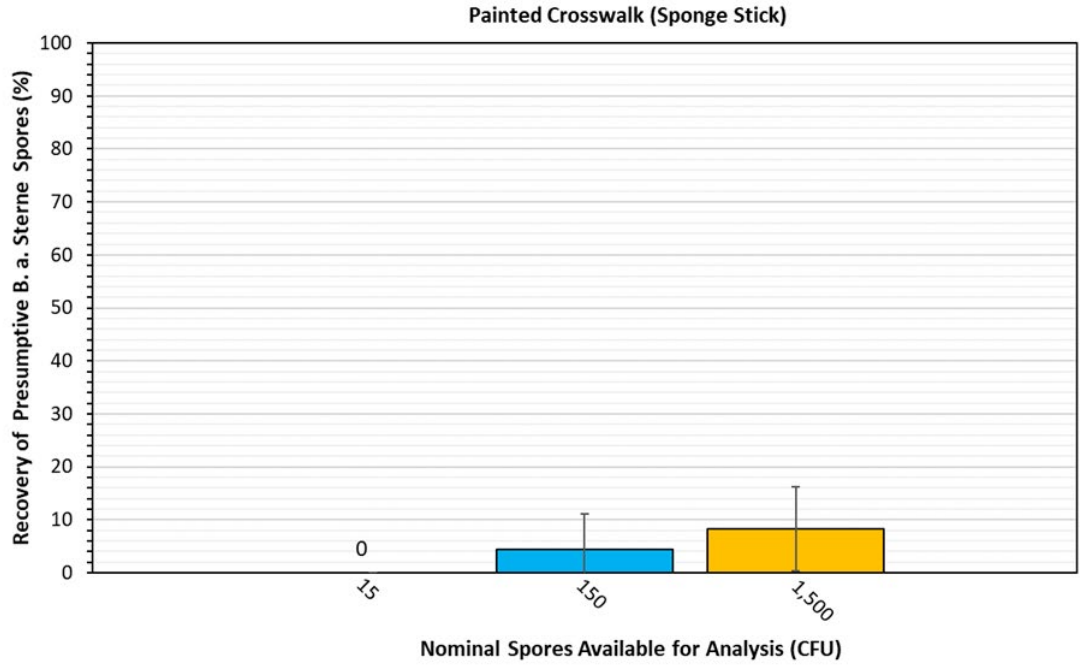

Figure 24. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Painted Crosswalk Sponge-Stick Samples Using SBA Medium

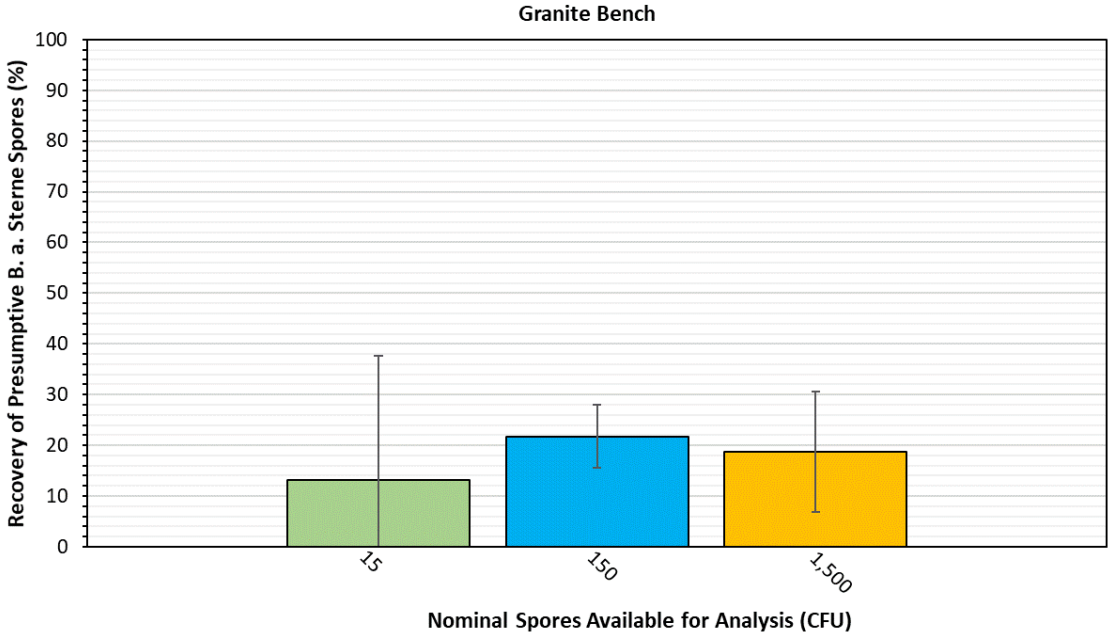

Figure 25. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Granite Bench Sponge-Stick Samples Using SBA Medium

A subset of colonies recovered were screened using real-time PCR assays targeting the chromosomal and pXO1 gene targets. A total of 229 colonies isolated from Sponge-Sticks were screened; of those colonies screened, 93% (213 correct) were confirmed as correctly identified.

Overall, background flora interfered with identification of presumptive *B. a.* Sterne from Street Grating (STGRAT) to a greater degree than the other surfaces. All STGRAT samples had background flora counts of greater than 83 colonies. A few representative images of SBA plates from STGRAT are shown in Figure 26.

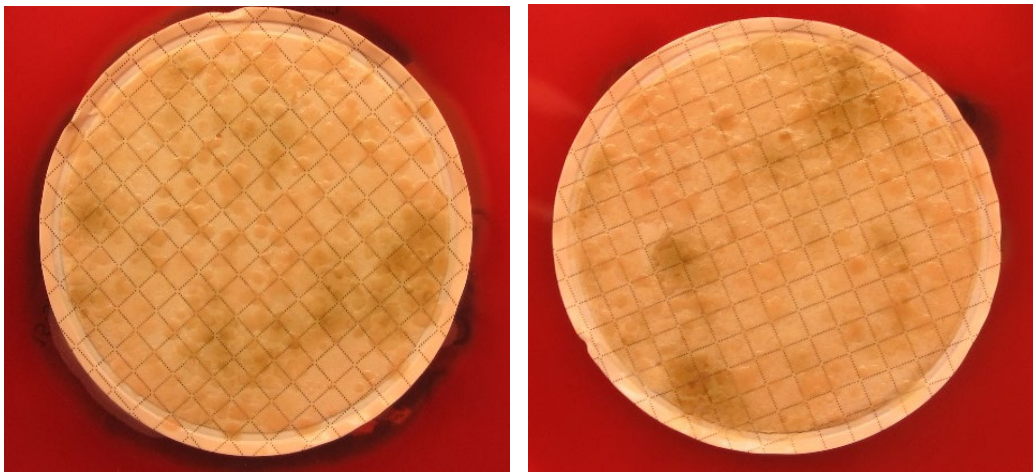

Figure 26. Sponge-Stick Samples from Street Grating Contained Background Flora that Interfered with Identification of *B. a.* Sterne Morphology to a Greater Degree Compared to Other Surfaces (both images were inoculated with 2 mL of extract).

The presence of material interfering with the analysis is not surprising considering that the Sponge-Sticks surface samples were noticeably dirty as shown in Figure 27.

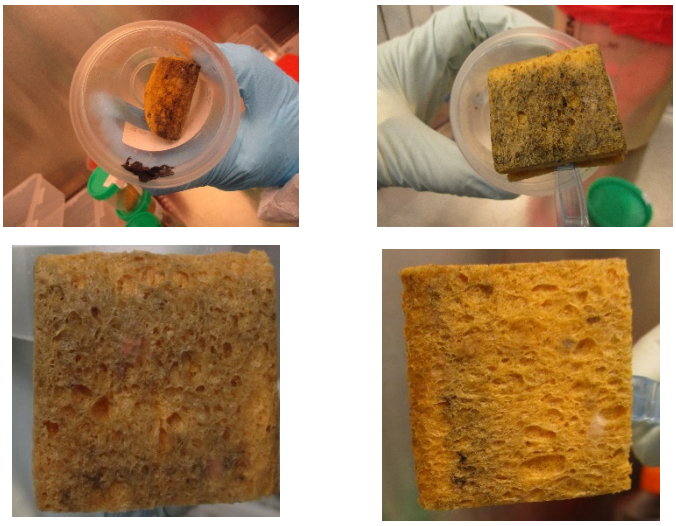

Figure 27. Sponge-Stick Samples: Subway Car Filter Grille (Top Left); Steps (Top Right); Crosswalk Signal (Bottom Left); Telephone Booth (Bottom Right)

3.1.2 Sponge-Stick TSB Enrichment

For Sponge-Sticks, there were instances where a colony with *B. a.* Sterne morphology was isolated from turbid TSB broth when streaked for isolation on SBA; those isolated colonies were screened using real-time PCR assays. In all cases the screened colonies were real-time PCR negative. For TSB broth suspension analysis using real-time PCR assays, 3 of the 21 Sponge-Stick samples that were screened resulted in average Ct values of ≤ 40 for at least 2 of 3 replicates for both chromosome and pXO1 real-time PCR assays (positive) and the other 18 samples were negative (Ct value of ≥ 40 for 2 of 3 replicates for both real-time PCR assays). All 3 positive samples were collected from the Street Grating surface, one each spiked with 30, 300, and 3,000 spore loads. The average Ct values from these three samples ranged from 36.5 to 40.3. For each of these samples, the Ct values generated from RV-PCR were lower than those generated during the TSB enrichment broth real-time PCR, indicating that the spore recovery method for Sponge-Sticks and RV-PCR enrichment of *B. a.* Sterne spores was more efficient than TSB enrichment of the extracted sponge. A summary of the PCR results of TSB-enriched samples is presented in Appendix S.

3.1.3 RV-PCR Method

A summary of the average and sample standard deviation of the RV-PCR ΔCt values for the detection of *B. a.* Sterne spores recovered from Sponge-Stick surface samples are presented in Table 5. The ΔCt results are plotted in Figure 28 through Figure 46 with each plot associated with 1 of the 19 specific surface samples. The summary table and associated plots follow the same column header and x-axis labeling convention as used for the presentation of culture results. Most notably, the nominal number of spores available for analysis of 15, 150, and 1,500 CFU are used; it represents the maximum number of spores available, assuming a 100% recovery efficiency and half the sample extract is available for RV-PCR analysis. The average quantity of spores determined available are presented in Table 5. The 0-spore-available condition is included in the plots because meaningful RV-PCR results can be obtained, unlike that for a recovery efficiency. The plots all depict an area shaded in red that is the region of a negative detection result and an area of green that is a positive detection result, delineated by both the chromosomal and pXO1 gene target ΔCt values having to be ≥ 9 to be a positive result. The RV-PCR results for each Sponge-Stick sample analyzed are presented in Appendix P.

RV-PCR analyses of all the Sponge-Stick samples with a nominal 150 or 1,500 *B. a.* Sterne spores available for analysis resulted in a positive RV-PCR response, ΔCt ≥ 9. The average ΔCt for the 1,500 nominal *B. a.* Sterne spore available condition exceeded 20 for all surface samples, other than that for the Street Grate, which had a value of 17 (see Figure 46). As shown in Figure 28 through Figure 46, RV-PCR ΔCt values were consistently the lowest for the nominal 15-spores-available condition and highest for the 1,500 nominal spore condition. All surface samples exhibited some suppression of ΔCt magnitude associated with the previously collected particulate material, which is apparent when the ΔCt magnitude for the nominal 15 *B. a.* Sterne spores available condition of the field blank samples (ΔCt = 25) is compared to that of the other surfaces' samples that typically have a ΔCt < 15, which suggests that the lower limit of detection of the RV-PCR method is near the nominal 15-spores-available condition for samples that also have ambient particulate matter also present.

RV-PCR had a positive response to all sponge wipe surface samples with a nominal 15 *B. a.* Sterne spores available for analysis condition, except for Street Grate and Painted Crosswalk surfaces. Street Grate and Painted Crosswalk each had at least one sample for which the RV-PCR response was negative (*B. a.* Sterne not detected) for the nominal 15-spores-

available condition, indicating the limit of detection of the method is being approached. Results showing that sample ΔCt standard deviations are relatively large and the greatest with the nominal 15-spores-available condition relative to those measured at the 150 and 1,500 spores available condition also suggests that the method detection limit is being approached at the 15-spore load.

There were instances where the 0-spike condition resulted in a measurable ΔCt value, but in no instances did ΔCt exceed 9, meaning that there were no RV-PCR false positive responses and that cross-contamination was not an issue.

Consistently, throughout all analyses, very good agreement (ΔCt differed by < 1 between the two gene targets) was obtained for the chromosomal and pXO1 gene targets for all sponge wipe surface samples and for all nominal spore loads.

Table 5. RV-PCR Analyses of Sponge-Stick Surface Samples for Detection of *B. a.* Sterne Spores Using Chromosomal and pXO1 Gene Targets (N = 3 Replicates for 0 Nominal Spike; N = 5 for 15, 150, and 1,500 Nominal Spike)

Surface Sample (Surface ID)	Spores Available for Analysis (CFU)		ΔCt (X ± σ)	
	Nominal[a]	Determined[b] (X ± σ)	Chromosomal Gene Target	pXO1 Gene Target
Floor Tile (FLTILE)	0	0	0	0
	15	14 ± 2.8	12.8 ± 2.2	12.6 ± 2.4
	150	140 ± 28	19.0 ± 2.8	19.1 ± 3.3
	1,500	1,400 ± 280	21.2 ± 2.0	21.2 ± 2.2
Floor (Concrete) (FLCON)	0	0	0	0
	15	14 ± 2.5	15.6 ± 1.3	15.7 ± 1.2
	150	140 ± 25	18.4 ± 2.5	18.2 ± 2.9
	1,500	1,400 ± 250	21.5 ± 1.0	21.6 ± 1.2
Step (Metal) (STEPS)	0	0	0	0
	15	12 ± 7.0	15.0 ± 1.9	14.9 ± 1.8
	150	140 ± 40	18.9 ± 2.7	18.9 ± 2.9
	1,500	1,500 ± 440	21.3 ± 1.6	21.2 ± 1.5
Wall Tile (WLTILE)	0	0	0	0.2 ± 0.2
	15	14 ± 4.2	14.3 ± 8.3	14.0 ± 8.2
	150	140 ± 42	22.5 ± 2.7	22.7 ± 2.9
	1,500	1,400 ± 420	22.4 ± 1.7	22.6 ± 2.1
Glass Window (GLSWIN)	0	0	1.0 ± 1.8	0.6 ± 0.8
	15	16 ± 3.9	18.9 ± 3.5	18.7 ± 3.3
	150	160 ± 41	23.0 ± 3.8	23.0 ± 3.9
	1,500	1,600 ± 410	25.9 ± 1.0	25.6 ± 1.6
Electronic Display Panel (Below Ground) (EDPAN(B))	0	0	0.7 ± 1.2	1.8 ± 2.1
	15	14 ± 7.3	20.8 ± 2.8	20.9 ± 2.8
	150	160 ± 36	23.1 ± 2.2	23.0 ± 2.3
	1,500	1,600 ± 360	24.9 ± 3.1	25.0 ± 3.1
Glass Panel (GLSPAN)	0	0	0	1.1 ± 2.0
	15	20 ± 5.9	16.6 ± 9.5	16.5 ± 9.5
	150	190 ± 55	23.9 ± 3.0	24.0 ± 2.8
	1,500	1,900 ± 550	24.1 ± 2.3	25.0 ± 1.2
Fluorescent Light Fixture (FLLFIX)	0	0	0	1.4 ± 1.5
	15	20 ± 5.3	17.9 ± 4.4	17.9 ± 4.3
	150	180 ± 56	19.2 ± 1.9	18.6 ± 2.7
	1,500	1,800 ± 560	23.2 ± 1.8	23.2 ± 1.9
Overhead Sign (OHSIGN)	0	0	0	0.5 ± 0.9
	15	10 ± 2.6	17.0 ± 2.5	17.3 ± 2.8
	150	100 ± 26	22.1 ± 1.9	22.1 ± 1.9
	1,500	1,000 ± 260	24.1 ± 2.2	23.9 ± 1.8

Table 5. RV-PCR Analyses of Sponge-Stick Surface Samples for Detection of *B. a.* Sterne Spores Using Chromosomal and pXO1 Gene Targets (N = 3 Replicates for 0 Nominal Spike; N = 5 for 15, 150, and 1,500 Nominal Spike) (Cont.)

Surface Sample (Surface ID)	Spores Available for Analysis (CFU)		ΔCt (X ± σ)	
	Nominal[a]	Determined[b] (X ± σ)	Chromosomal Gene Target	pXO1 Gene Target
Metro Card Machine (MCMACH)	0	0	0.6 ± 1.1	0.4 ± 0.7
	15	91 ± 1.6	14.9 ± 3.8	14.9 ± 3.9
	150	100 ± 23	18.7 ± 1.2	18.8 ± 1.3
	1,500	1,000 ± 230	21.3 ± 1.7	21.5 ± 1.6
Subway Car Filter Grille (SCGRIL)	0	0	0	0.5 ± 0.9
	15	15 ± 3.5	14.3 ± 2.5	14.3 ± 2.5
	150	140 ± 34	17.3 ± 5.4	17.6 ± 5.2
	1,500	1,500 ± 350	21.0 ± 2.6	21.0 ± 2.8
Control Field Blank (FLDBLK)	0	0	2.5 ± 4.3	2.5 ± 4.3
	15	12 ± 3.0	26.5 ± 0.9	25.7 ± 1.8
	150	130 ± 27	25.7 ± 0.3	25.6 ± 0.4
	1,500	1,300 ± 230	25.6 ± 0.6	24.9 ± 1.6
Sidewalk (Concrete) (SWCON)	0	0	1.5 ± 2.7	1.4 ± 2.4
	15	15 ± 2.7	14.8 ± 1.8	15.3 ± 2.2
	150	150 ± 27	18.7 ± 3.1	19.2 ± 3.1
	1,500	1,500 ± 270	22.5 ± 1.3	22.9 ± 1.2
Electronic Display Panel (Above Ground) (EDPAN(A))	0	0	0.3 ± 0.6	1.5 ± 1.5
	15	15 ± 3.2	17.8 ± 5.3	17.8 ± 5.2
	150	150 ± 32	22.3 ± 1.7	22.3 ± 1.8
	1,500	1,500 ± 320	25.0 ± 1.6	25.1 ± 1.7
Crosswalk Signal (CWSIGN)	0	0	0.6 ± 1.1	0.7 ± 0.6
	15	15 ± 3.9	17.3 ± 2.8	17.5 ± 2.8
	150	150 ± 43	20.2 ± 3.6	20.2 ± 3.5
	1,500	1,500 ± 430	24.2 ± 2.0	24.2 ± 2.0
Telephone Booth (TELEBO)	0	0	0	0
	15	17 ± 1.8	18.3 ± 3.8	18.4 ± 3.6
	150	170 ± 18	19.8 ± 3.2	19.7 ± 3.7
	1,500	1,700 ± 180	22.5 ± 0.9	22.6 ± 0.8
Street Grate (STGRAT)	0	0	2.5 ± 4.3	2.2 ± 4.1
	15	12 ± 6.3	8.5 ± 1.7	9.7 ± 2.2
	150	140 ± 28	14.2 ± 2.0	14.3 ± 1.5
	1,500	1,400 ± 280	16.6 ± 2.4	17.5 ± 2.6
Crosswalk (Painted) (CWPNTD)	0	0	0	0
	15	8.8 ± 4.5	11.4 ± 7.1	11.8 ± 4.9
	150	110 ± 12	18.4 ± 4.2	18.5 ± 4.1
	1,500	1,100 ± 120	19.7 ± 3.8	19.9 ± 3.8
Granite Bench (GRNBEN)	0	0	0.5 ± 0.9	0.6 ± 1.0
	15	11 ± 2.7	14.9 ± 4.2	14.1 ± 4.5
	150	130 ± 42	19.3 ± 1.7	19.3 ± 1.8
	1,500	1,300 ± 420	21.6 ± 2.4	21.6 ± 2.1

(a) Nominally one-half of the target spore load on the filter and assuming 100% recovery of spores.

(b) Based on the spiking suspension titer measured each test trial, 100% recovery efficiency, and one-half of extract used for RV-PCR analysis.

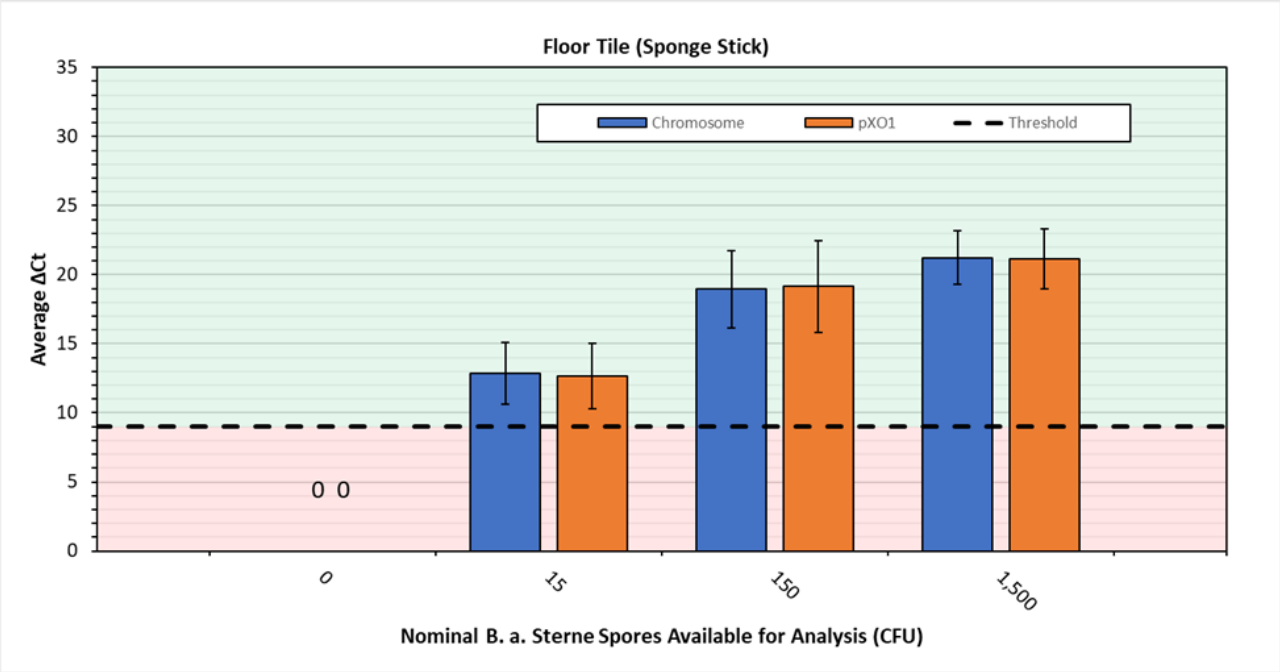

Figure 28. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Floor Tile Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

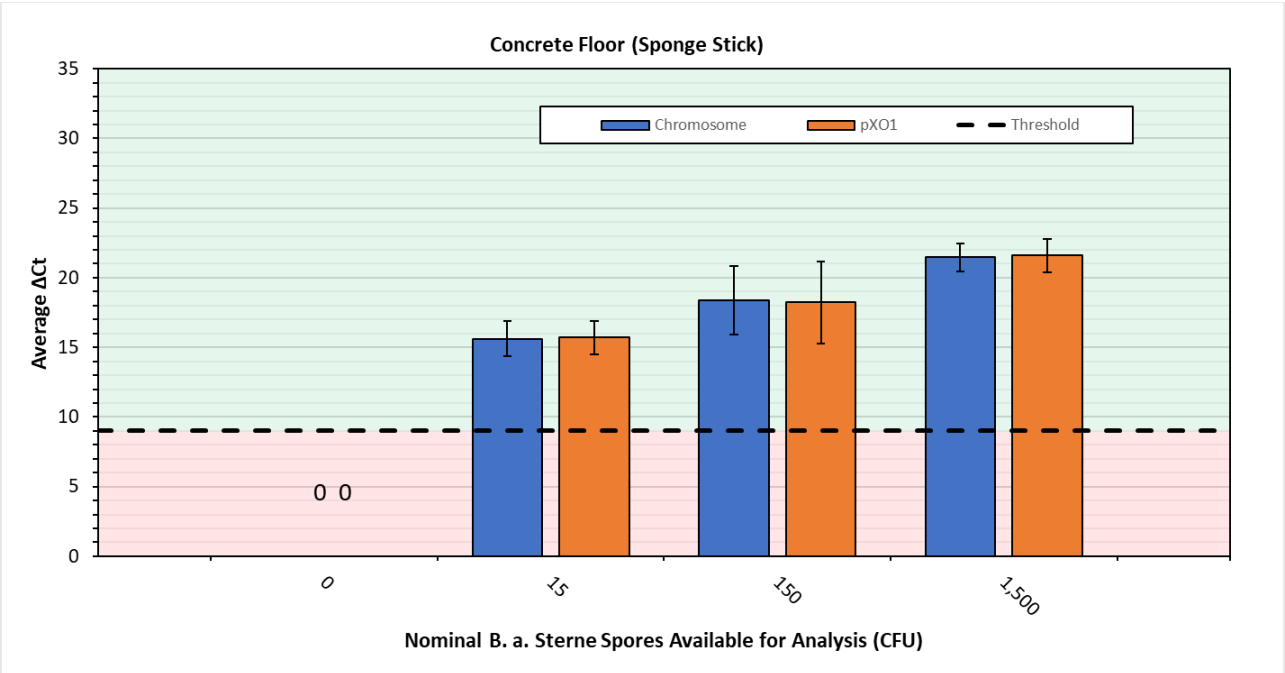

Figure 29. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Concrete Floor Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

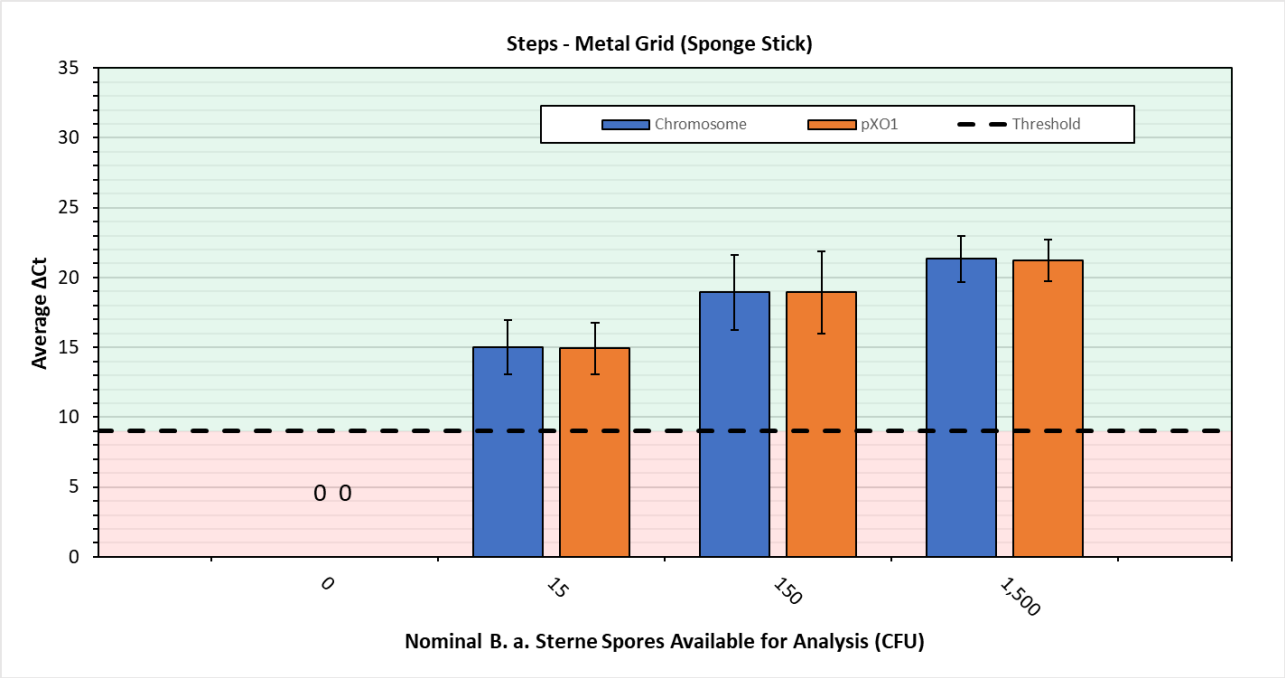

Figure 30. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Steps (Metal) Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

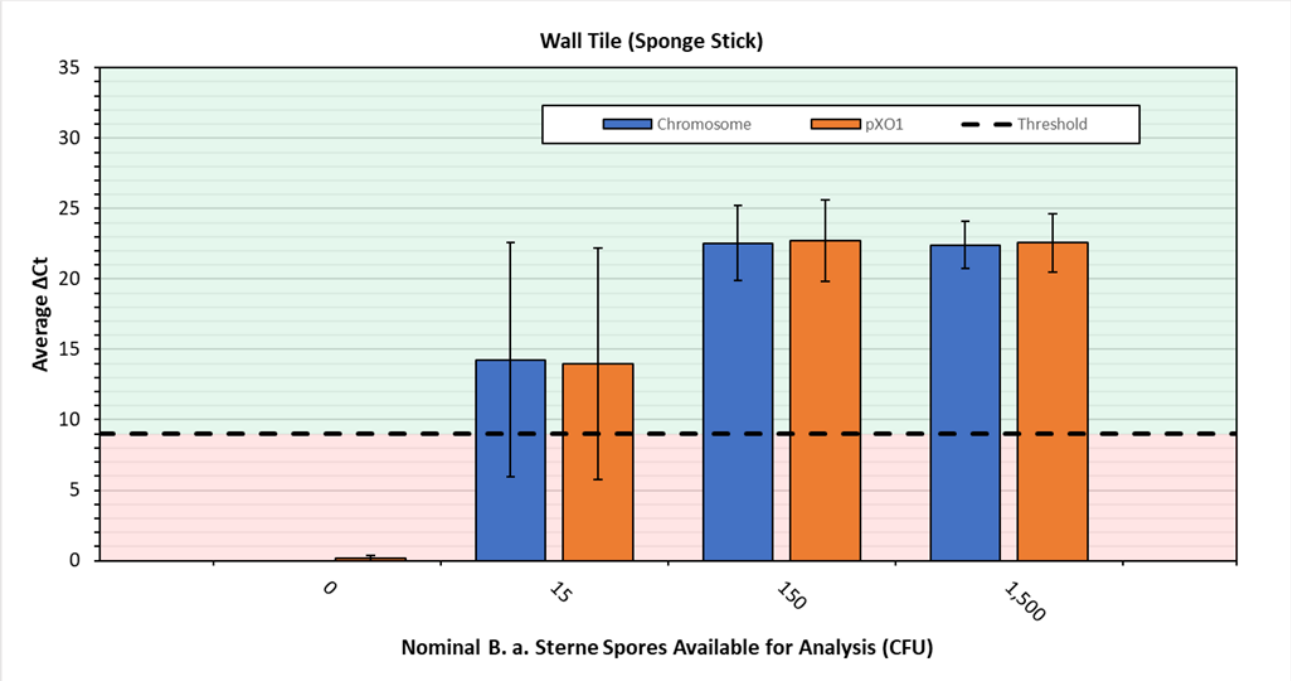

Figure 31. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Wall Tile Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

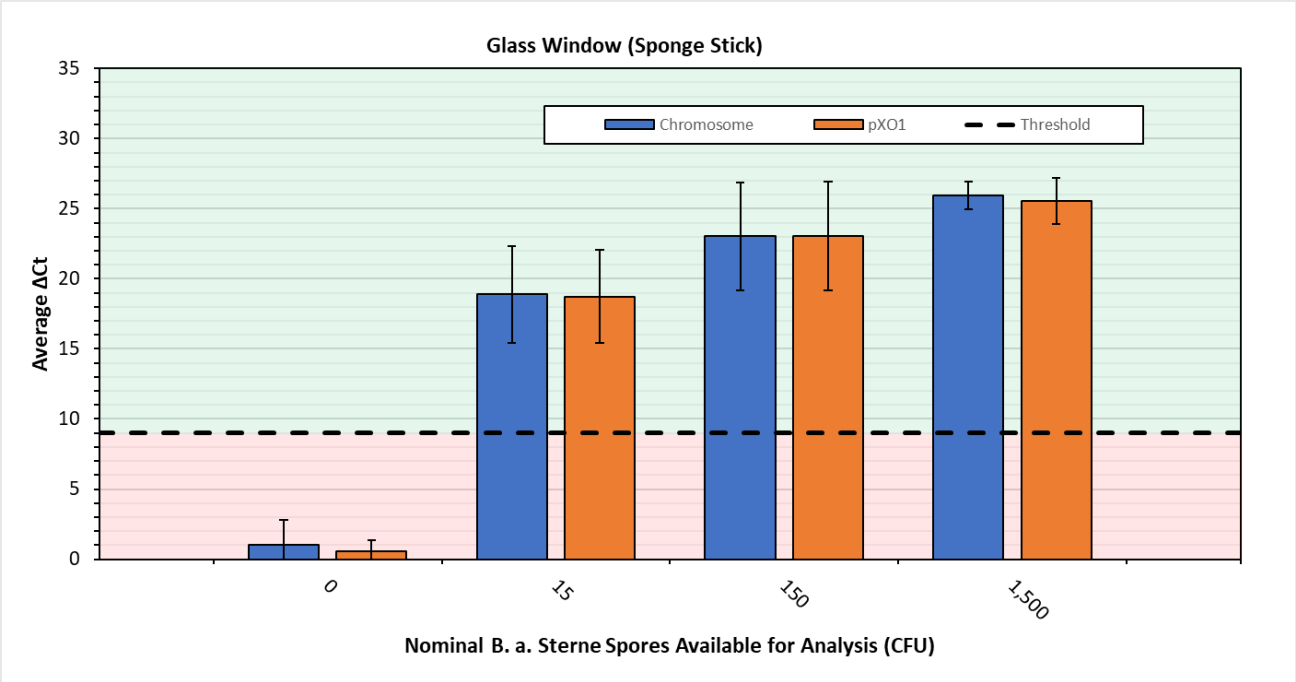

Figure 32. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Glass Window Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

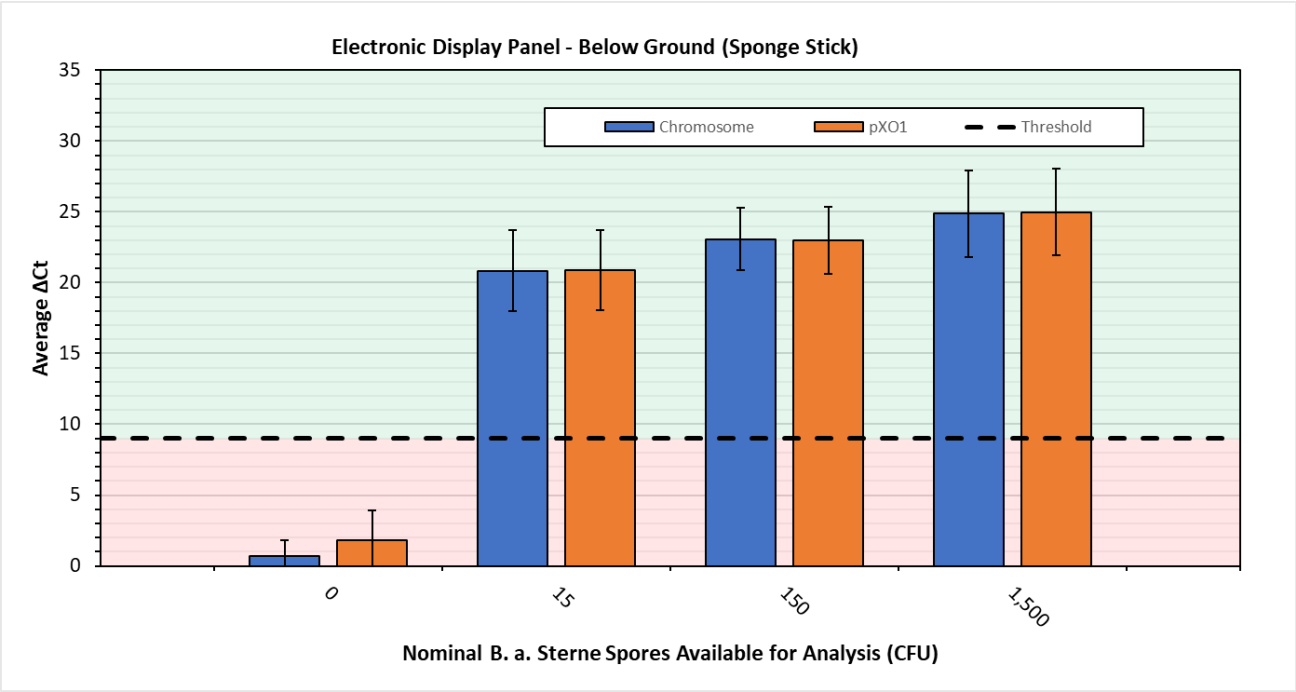

Figure 33. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Electronic Display Panel (Below Ground) Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

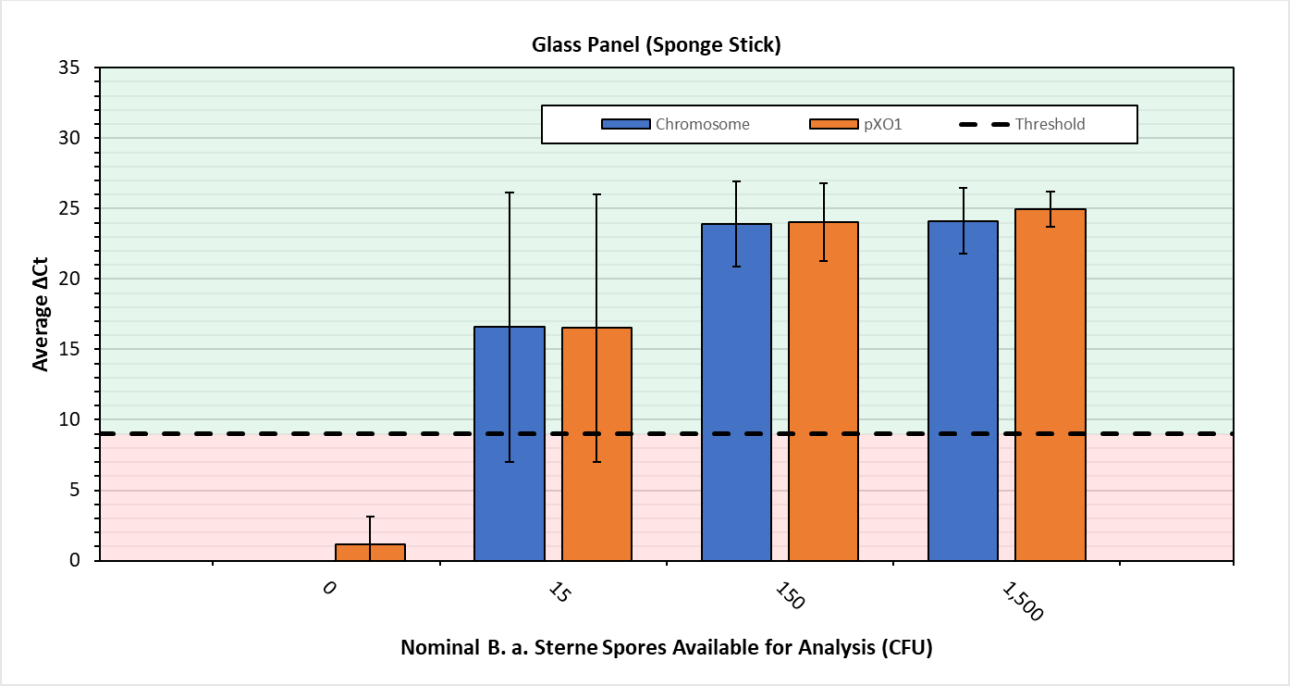

Figure 34. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Glass Panel Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

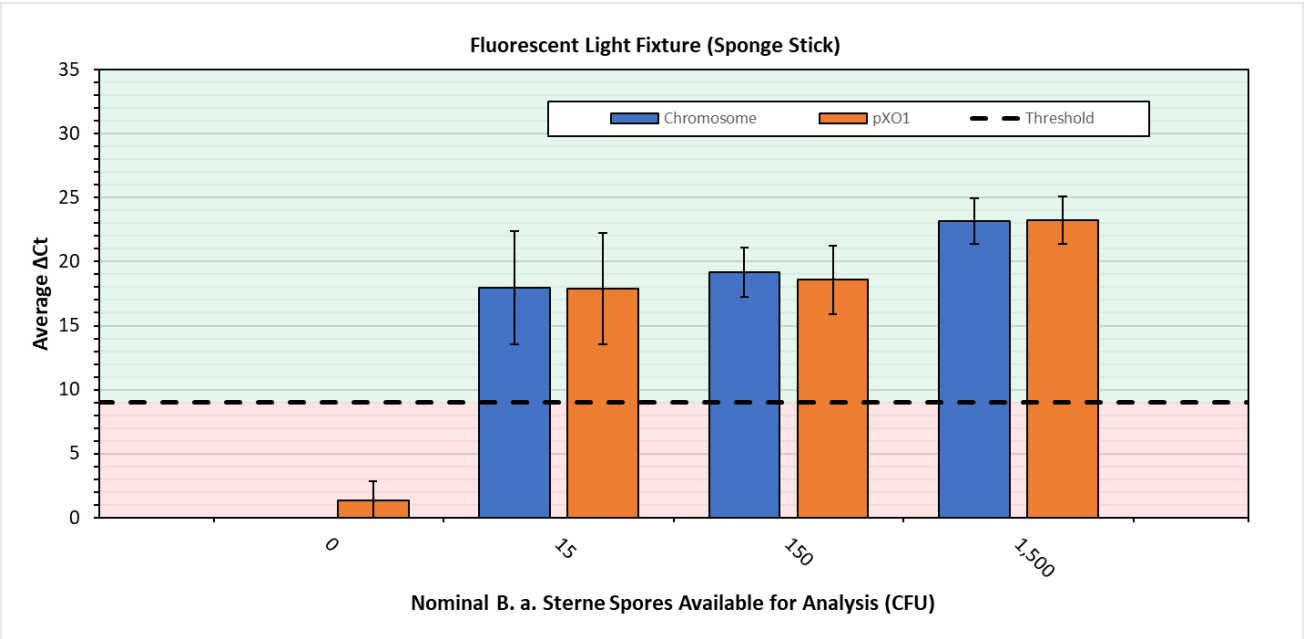

Figure 35. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Fluorescent Light Fixture Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

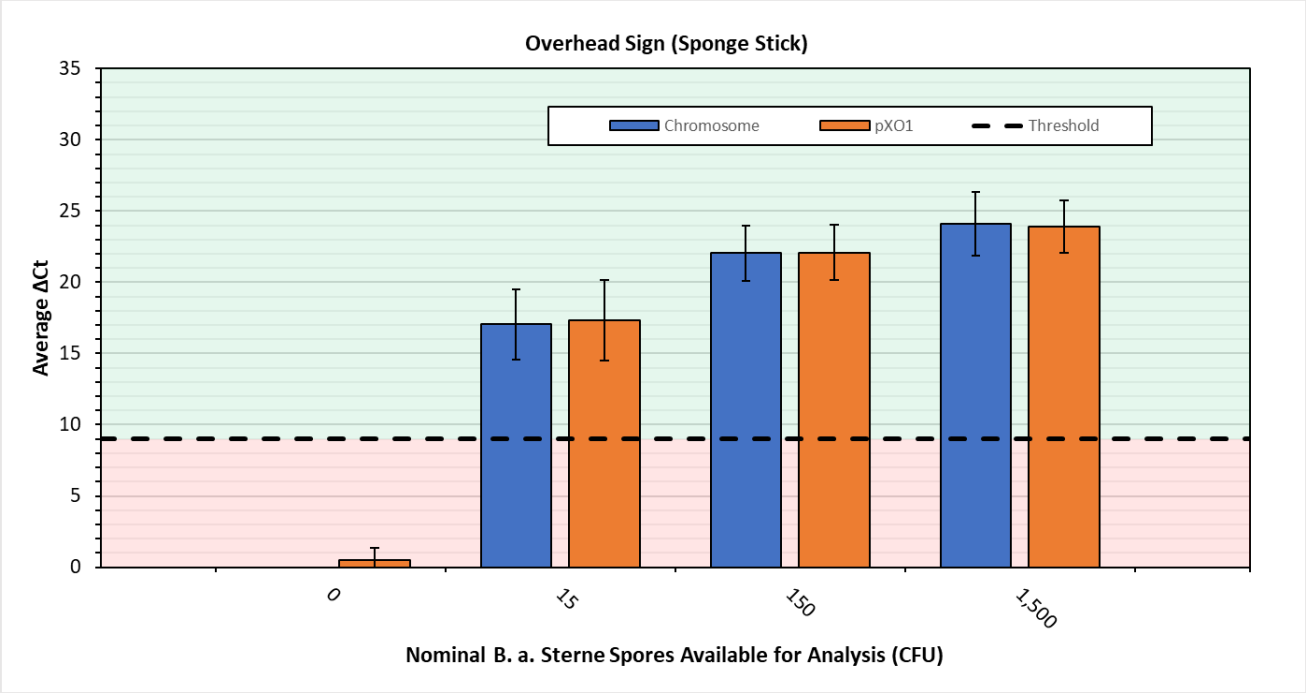

Figure 36. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Overhead Sign Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

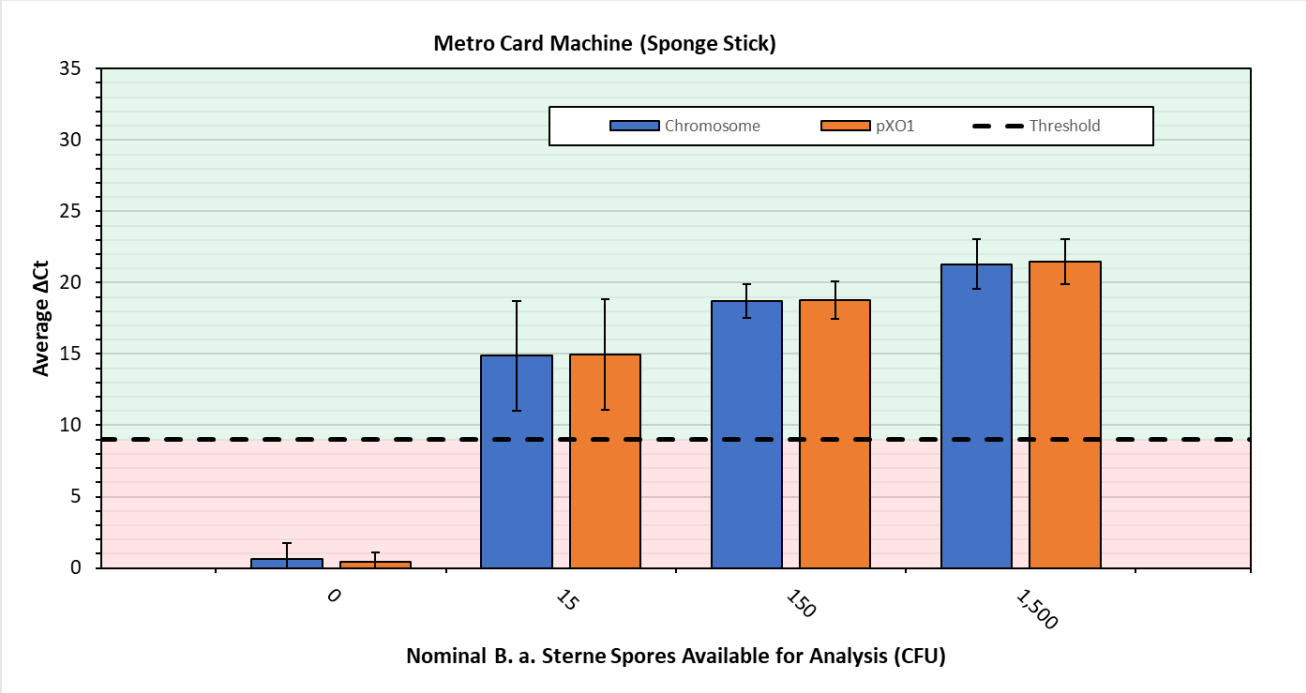

Figure 37. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Metro Card Machine Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

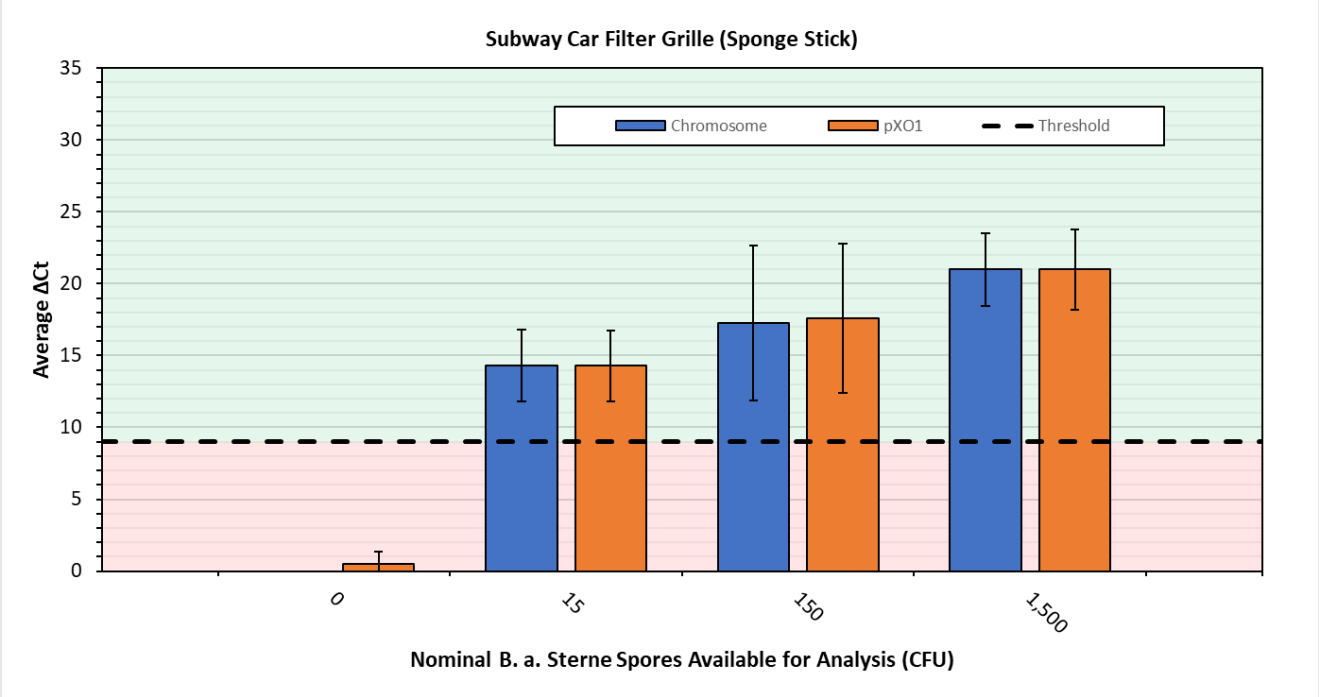

Figure 38. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Subway Car Filter Grille Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

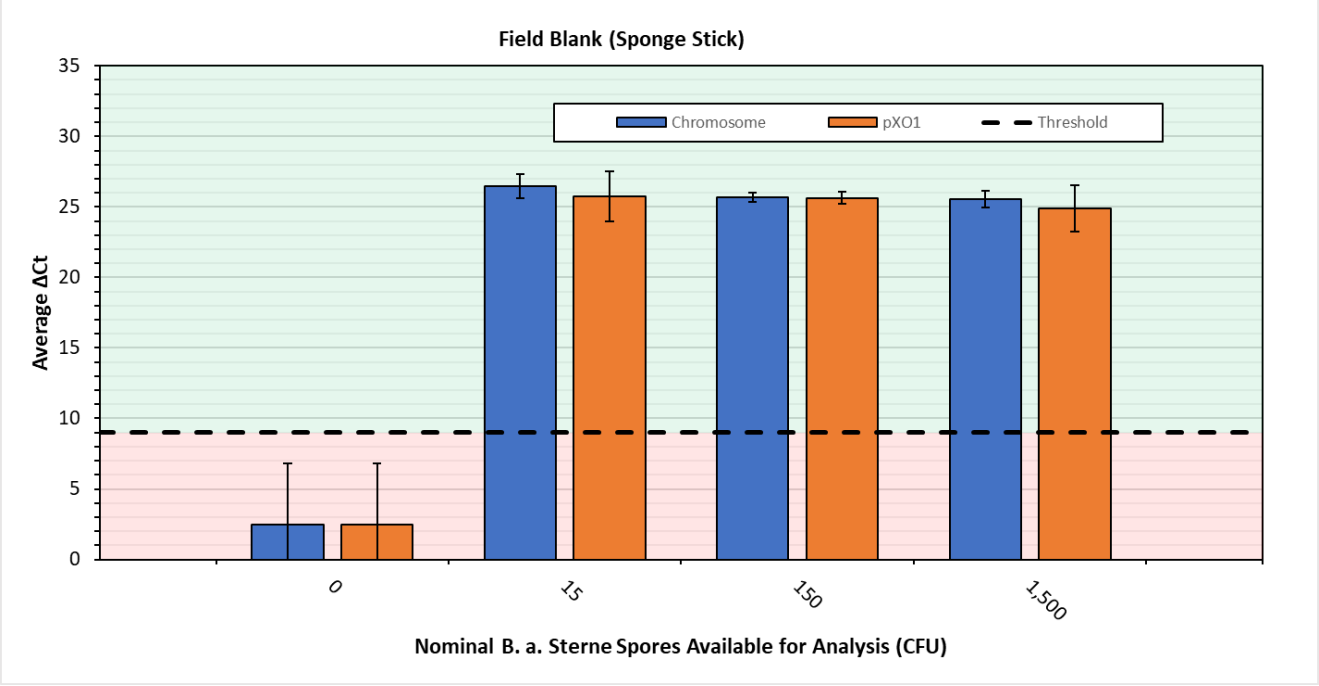

Figure 39. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Field Blank Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

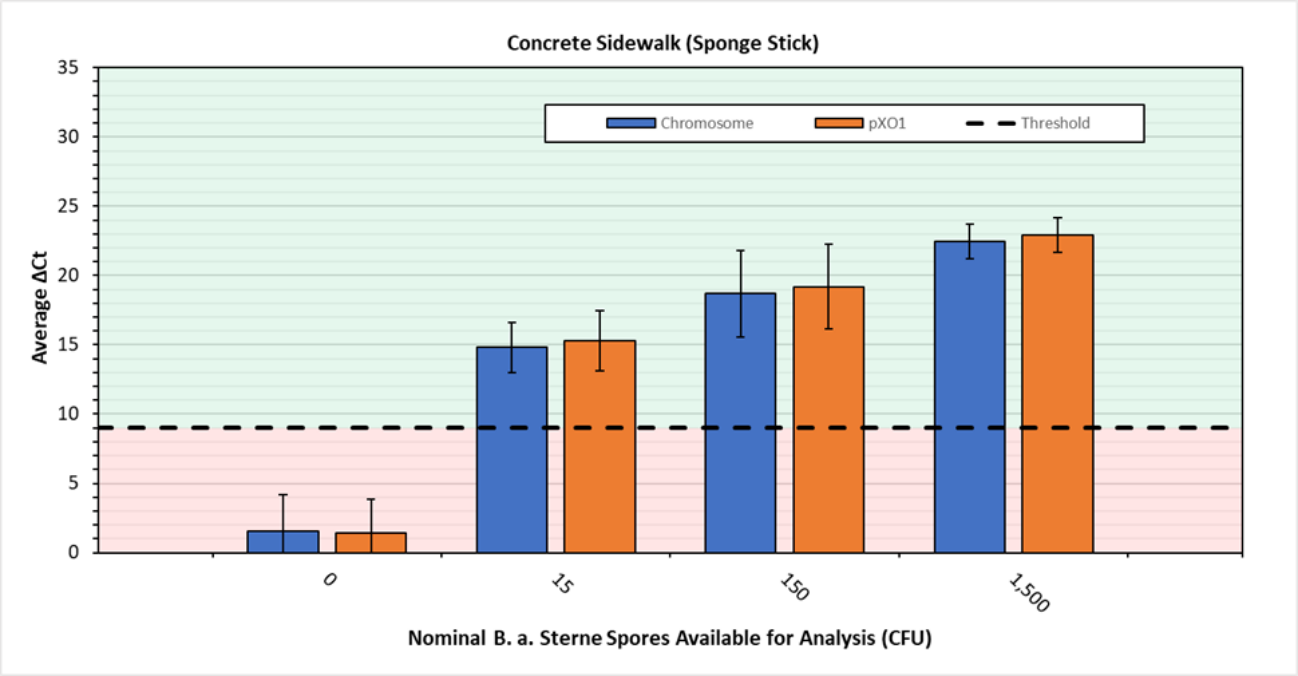

Figure 40. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Concrete Sidewalk Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

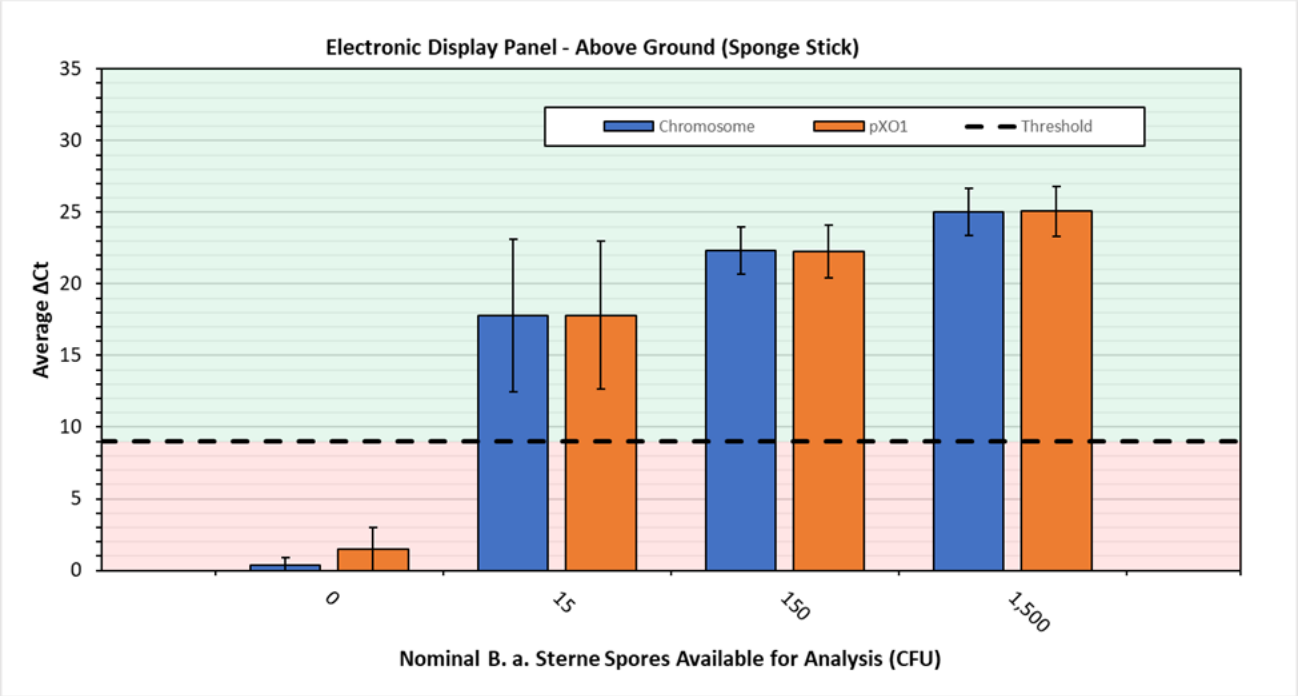

Figure 41. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Electronic Display Panel (Above Ground) Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

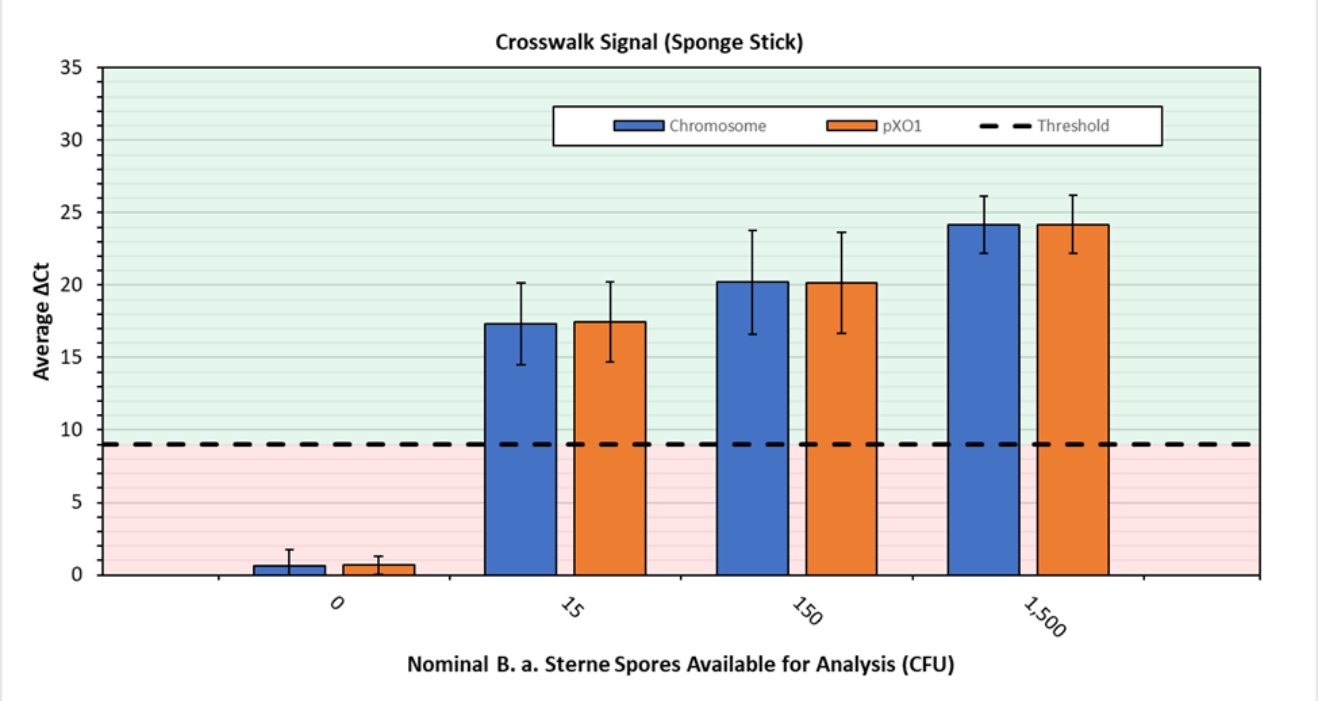

Figure 42. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Crosswalk Signal Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

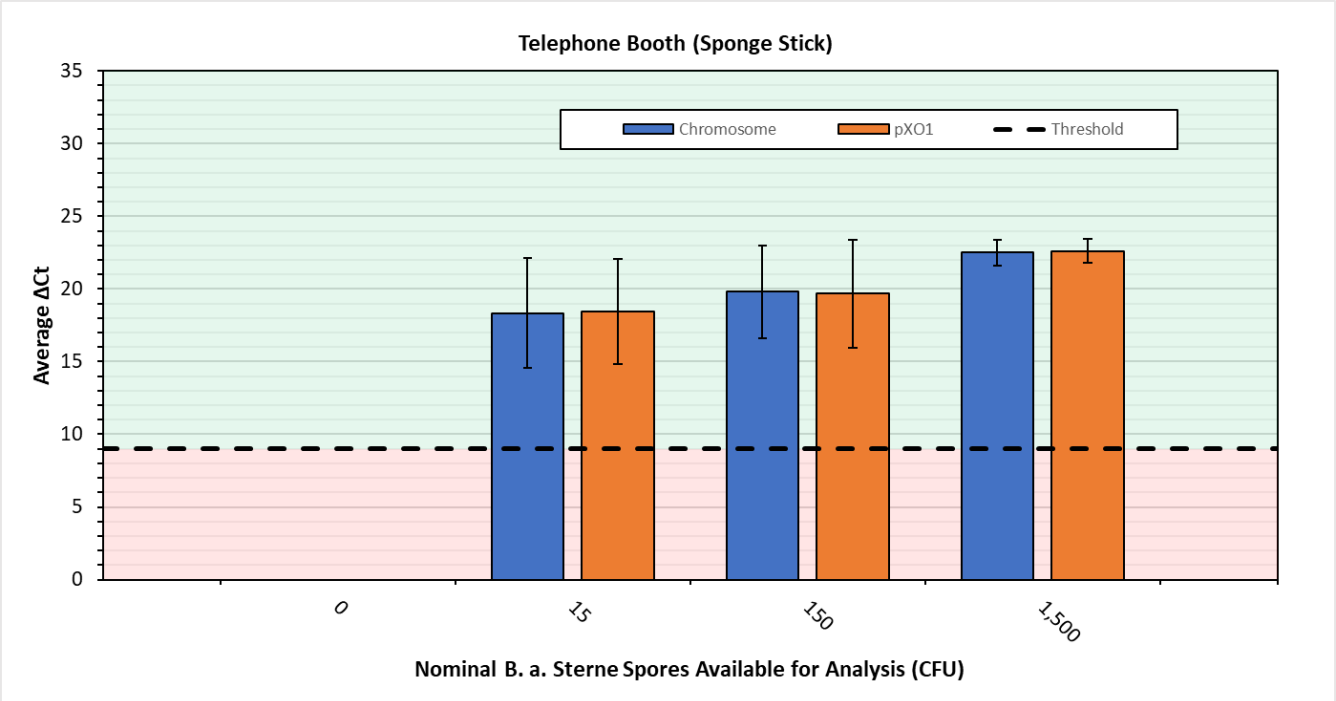

Figure 43. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Telephone Booth Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

Figure 44. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Street Grate Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

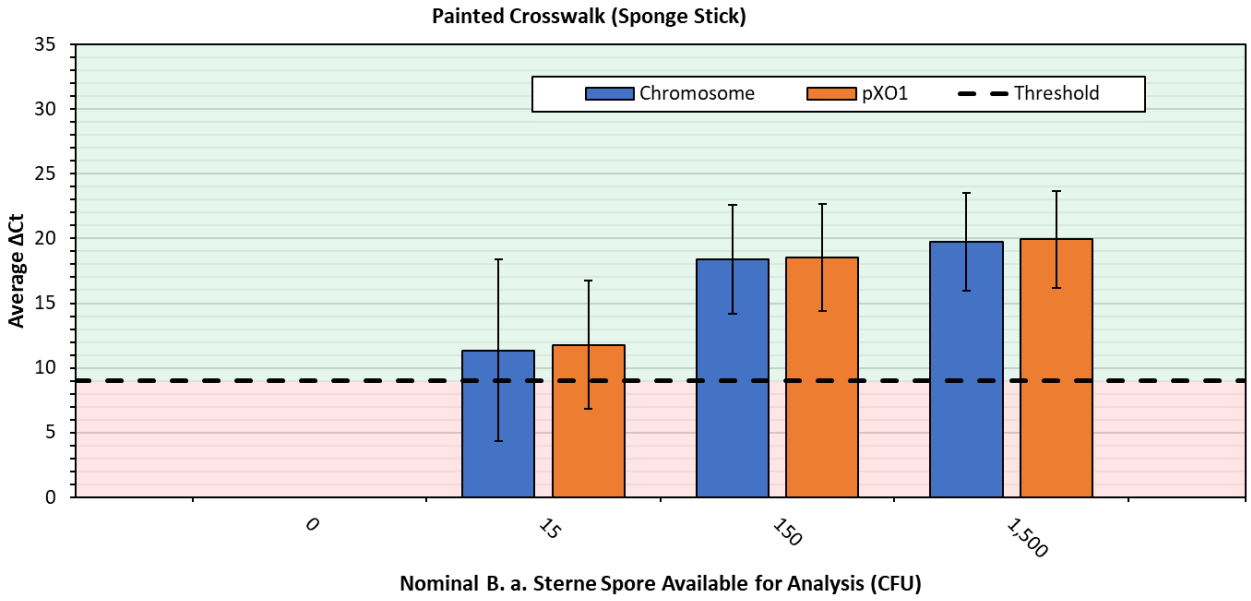

Figure 45. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Painted Crosswalk Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

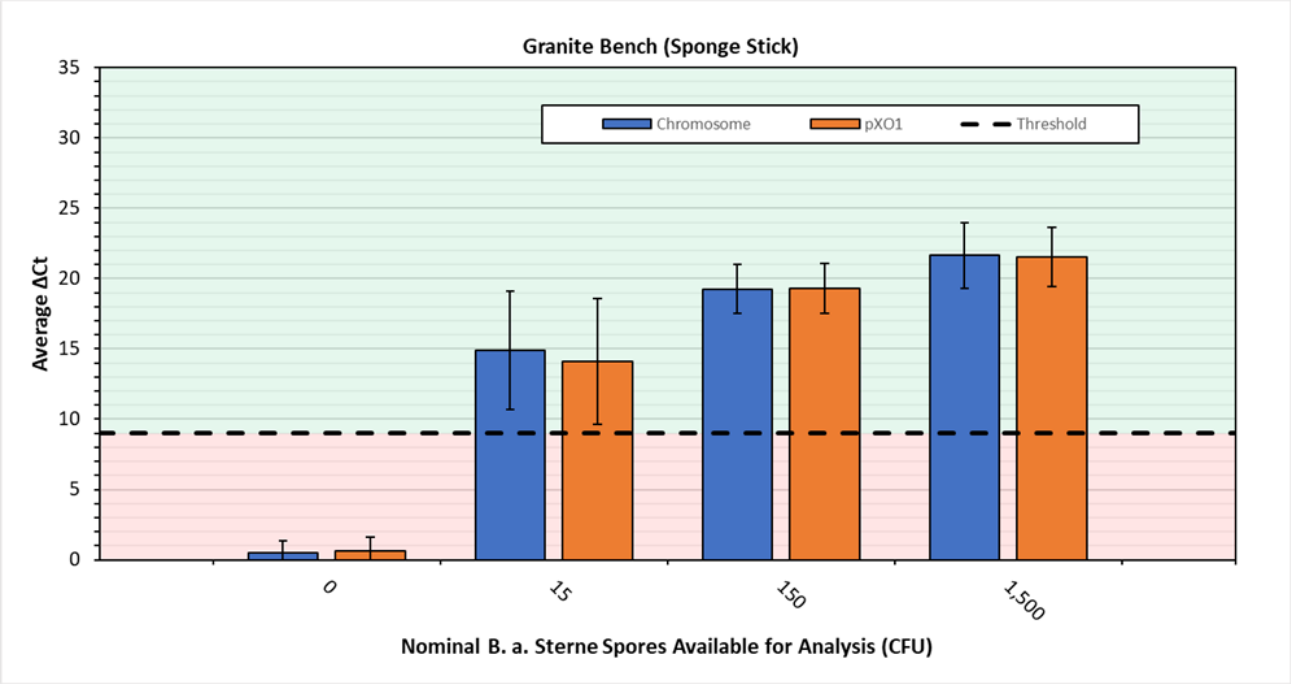

Figure 46. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Granite Bench Sponge-Stick Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

3.2 Vacuum Filter Cassette Analyses Results

3.2.1 Culture Method

A summary of the average and standard deviation of the measured recovery efficiencies of presumptive *B. a.* Sterne spores recovered from VFC samples spiked with *B. a.* Sterne spores and using SBA medium to culture are presented in Table 6. The determined number of spores available and the number of presumptive *B. a.* Sterne spores recovered are tabulated along with the nominal quantity of spores available for analysis (15, 150 and 1,500 CFU/filter sample). The quantity of presumptive *B. a.* Sterne colonies reported in the table is half of the actual total recovered because in the context of the tables, only half of the extract samples was made available for analysis. The quantity of presumptive *B. a.* Sterne colonies for each Sponge-Stick sample used in the percent recovery calculations are reported in Appendix Q.

The recovery efficiencies are plotted in Figure 48 through Figure 54 one plot for each sampled location using VFCs. Note, a percent recovery is not tabulated nor plotted for the 0-spore spike condition since, by definition, a meaningful recovery efficiency cannot be calculated, even

though there could have been presumptive *B. a.* Sterne colonies counted based on colony morphology.

The recovery efficiencies of presumptive *B. a.* Sterne from VFCs were generally low (< 10%) for all surfaces sampled, which is attributed primarily to spores being retained on the MCE filter substrate in the VFC and/or to some extent on the particulate matter previously collected on the VFC. Instances where the average recovery efficiency exceeded 10% were associated with a large standard deviation, which was the result of few (< 10 colonies) being counted. The field blank recovery efficiencies were 0, 4, and < 1% for nominal 15-, 150-, and 1,500-spore-available condition, respectively, suggesting the spiked spores may be retained on the VFC MCE substrate.

Whether the instances of the recovery efficiency from VFCs being less than 100% were due to less than complete physical recovery of the spore or other physical loss mechanisms such as retention on processing containers or interference of growth due to the presence of grime or competing flora, was not resolved. It is also noted that spore/filter surface interactions may influence the percent recovery measured, which could be affected by the spore spiking method. The application of spores using droplets of a stock suspension may both assist or hinder the ability to physically recover the spores from VFCs. Spores collected as an aerosol as expected during normal field operation may adhere to the filter substrate more strongly than the majority of spores present when applied as a droplet of suspension. When spores are applied as a droplet of spore suspension, the spores may more readily disperse into the extract solution either due to being a large agglomerate or weakly adhered to a surface. Conversely, spores collected as an individual entity may adhere to the VFC substrate more strongly and be more difficult to physically recovery. For those reasons, it would be recommended to consider additional research to conduct similar analyses as performed here, but “spike” the *B. a.* Sterne by aerosolizing and collecting onto the filter via air sampling.

Table 6. Recovery Efficiencies for Presumptive *B. a.* Sterne Spores from Vacuum Filter Cassette Surface Samples Cultured in SBA Medium

Surface Sample (Surface ID)	Sample Reps	Spores Available for Analysis (CFU)		Spore Recovery (CFU) (X ± σ)[(c)]	Spore Recovery Efficiency (%) (X ± σ)[(d)]
		Nominal[(a)]	Determined (X ± σ)[(b)]		
Floor Concrete (FLCON)	3	0	0	9.2 ± 16	N/A
	5	15	17 ± 5	5.7 ± 6.7	38 ± 51
	5	150	170 ± 50	3.0 ± 4.8	2.2 ± 3.6
	5	1,500	1,700 ± 500	140 ± 270	9.8 ± 20
Steps (Metal) (STEPS)	3	0	0	0	N/A
	5	15	16 ± 5.6	22 ± 31	160 ± 220
	5	150	160 ± 56	45 ± 73	31 ± 51
	5	1,500	1,600 ± 560	3.3 ± 4.9	0.2 ± 0.3
Carpet (CARPET)	3	0	0	1.8 ± 3.2	N/A
	5	15	16 ± 5.2	3.3 ± 7.4	23 ± 51
	5	150	160 ± 52	24 ± 26	17 ± 19
	5	1,500	1,600 ± 520	5.5 ± 9.5	0.3 ± 0.4
Subway Car Filter (SCFILT)	3	0	0	0	N/A
	5	15	17 ± 4.8	24 ± 24	150 ± 160
	5	150	170 ± 48	33 ± 49	21 ± 31
	5	1,500	1,700 ± 480	4.8 ± 7.9	0.3 ± 0.5
Sidewalk (Concrete) (SWCON)	3	0	0	0	N/A
	5	15	14 ± 2.3	1.9 ± 2.6	12 ± 17
	5	150	140 ± 23	1.5 ± 2.4	1.0 ± 1.5
	5	1,500	1,400 ± 230	13 ± 11	1.0 ± 0.8
Pavement (PAVEMT)	3	0	0	0	N/A
	5	15	14 ± 0.9	5.5 ± 12	41 ± 91
	5	150	140 ± 9.1	28 ± 55	20 ± 41
	5	1,500	1,400 ± 91	8.8 ± 6.4	0.7 ± 0.5
Control Field Blank (FLDBLK)	3	0	0	0	N/A
	5	15	12 ± 2.5	0 ± 0	0.0 ± 0.0
	5	150	120 ± 25	4.1 ± 4.6	3.8 ± 4.7
	5	1,500	1,200 ± 250	4.4 ± 2.8	0.4 ± 0.3

(a) Nominally one-half of the target spore load on the filter and assuming 100% recovery of spores.

(b) Based on the spiking suspension titer measured for each test trial, 100% recovery efficiency, and one-half of extract used for culture analysis.

(c) Presumptive *B. a.* Sterne colonies based on morphology and one-half of extract used for culture analysis.

(d) Calculated using the actual spore loading on each filter and presumptive *B. a.* Sterne spores recovered on each VFC sample.

Most notable is the relatively large sample standard deviations and recovery efficiencies exceeding 100% associated with the nominal 15-spores-available condition that are attributed to few *B. a.* spores recovered and/or impact of background flora that could bias the presumptive *B. a.* Sterne spore count high or low.

The Subway Car Filters (SCFILT) appeared (by visual observation) to be the dirtiest of the VFC filters and had an abundance of background flora that complicated the identification and quantification of recovered *B. a.* Sterne. Figure 47 contains photographs of representative SCFILT samples at various stages, from sample receipt, to spore recovery and culture plates qualitatively illustrating the dirtiness of the samples and the impact on the culture plates to quantifying *B. a.* Sterne.

Figure 47. Subway Car Filter (SCFILT) Representative Images, Sample Receipt, Addition of Extraction Buffer, and Culture Growth on SBA Filter Spiked with 30, 300, or 3,000 Spores (from left to right, respectively)

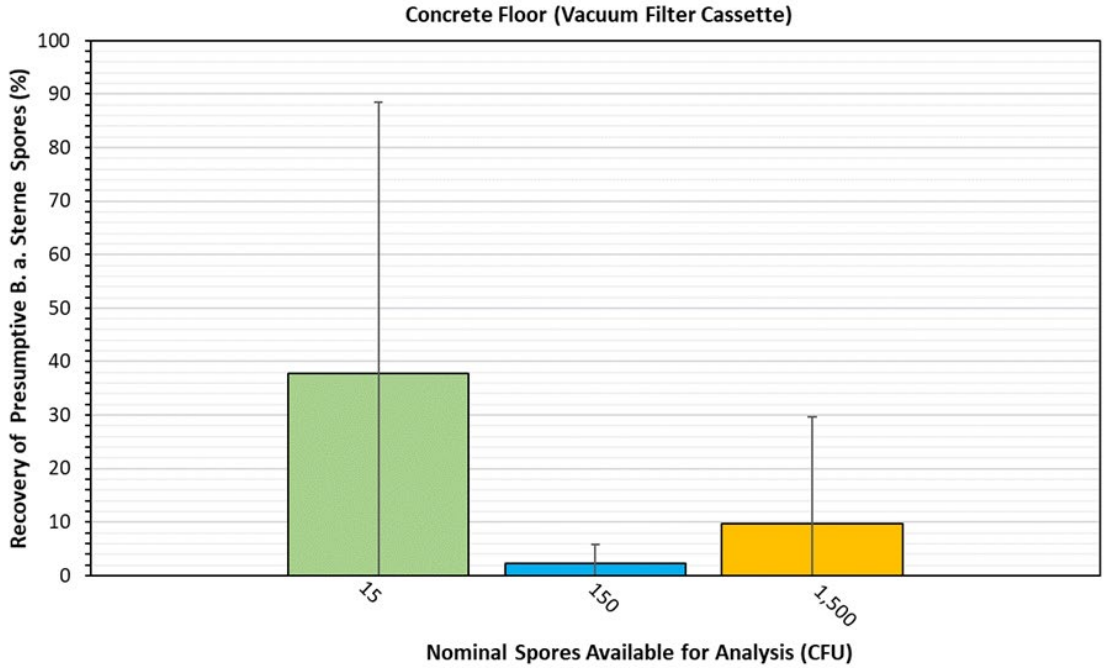

Figure 48. Percent Recovery Efficiencies (Average ± One Standard Deviation of N =5 Replicates) of Presumptive *B. a.* Sterne Spores from Concrete Floor Vacuum Filter Cassette Samples Using SBA Medium

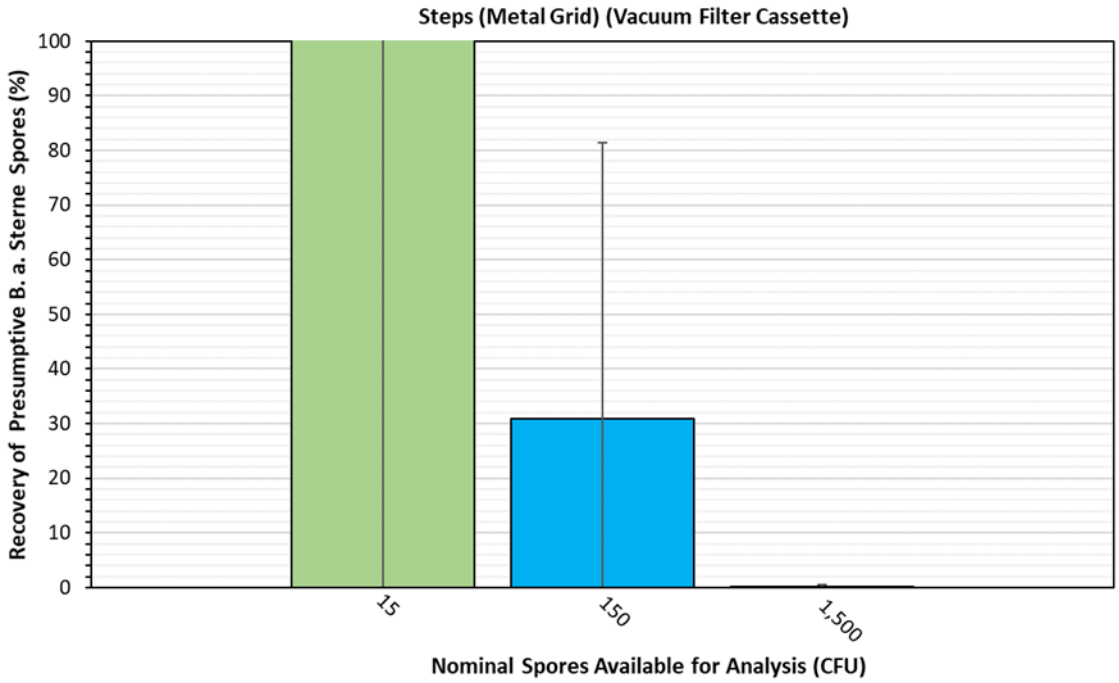

Figure 49. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Steps (Metal) Vacuum Filter Cassette Samples Using SBA Medium

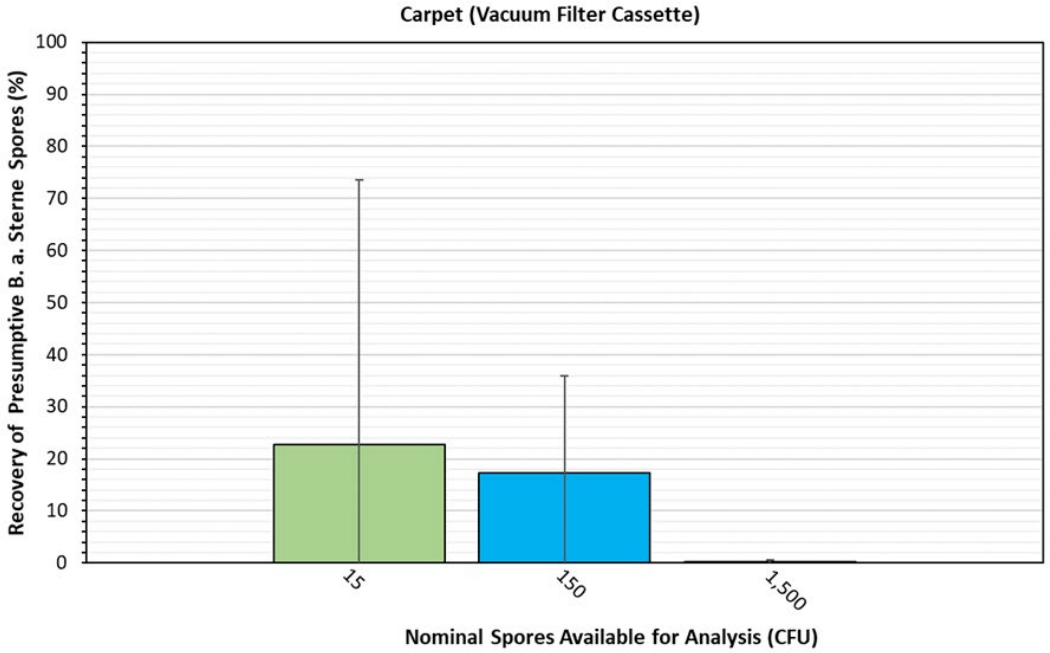

Figure 50. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Carpet Vacuum Filter Cassette Samples Using SBA Medium

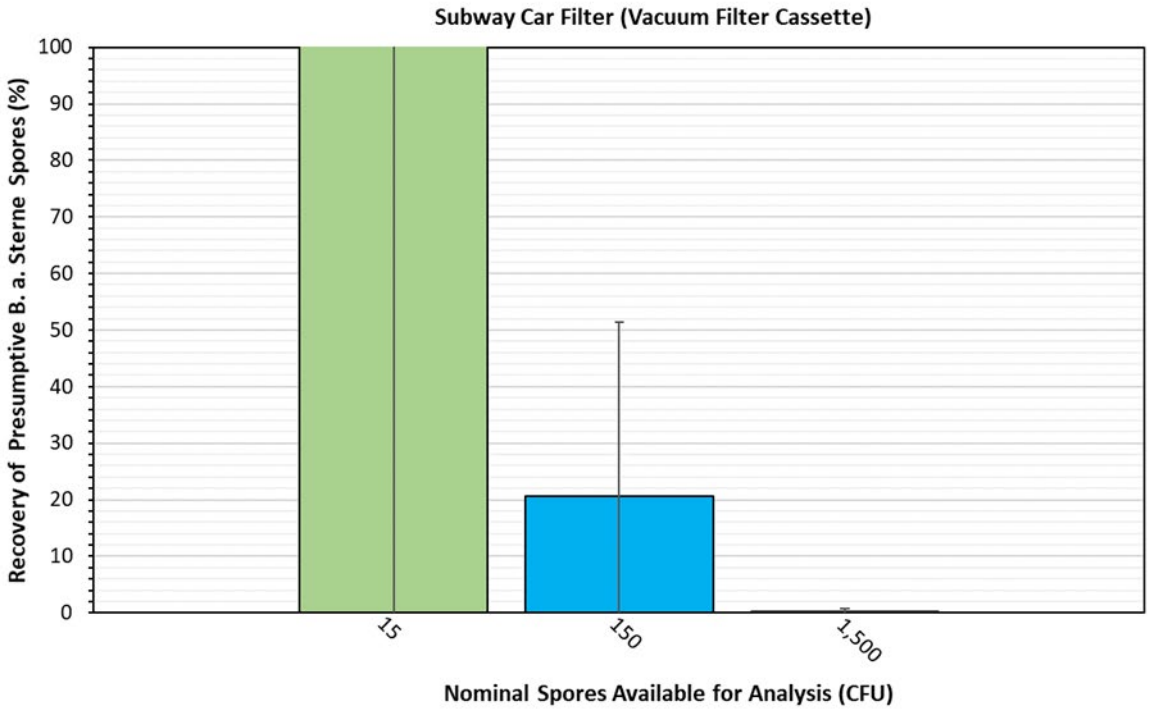

Figure 51. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Subway Car Filter Vacuum Filter Cassette Samples Using SBA Medium

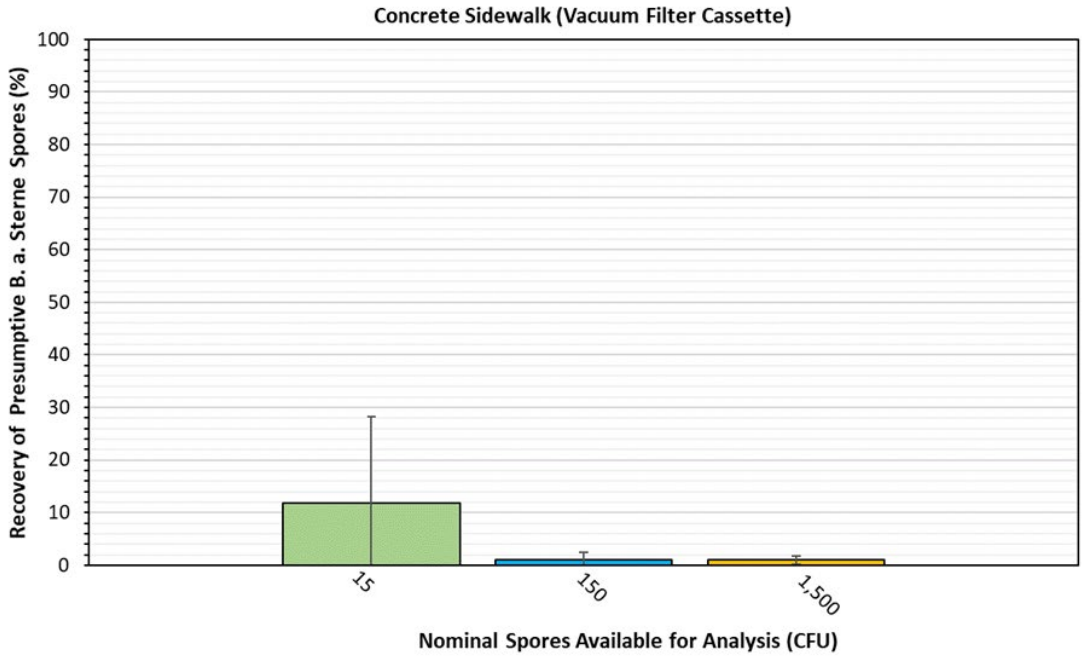

Figure 52. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Concrete Sidewalk Vacuum Filter Cassette Samples Using SBA Medium

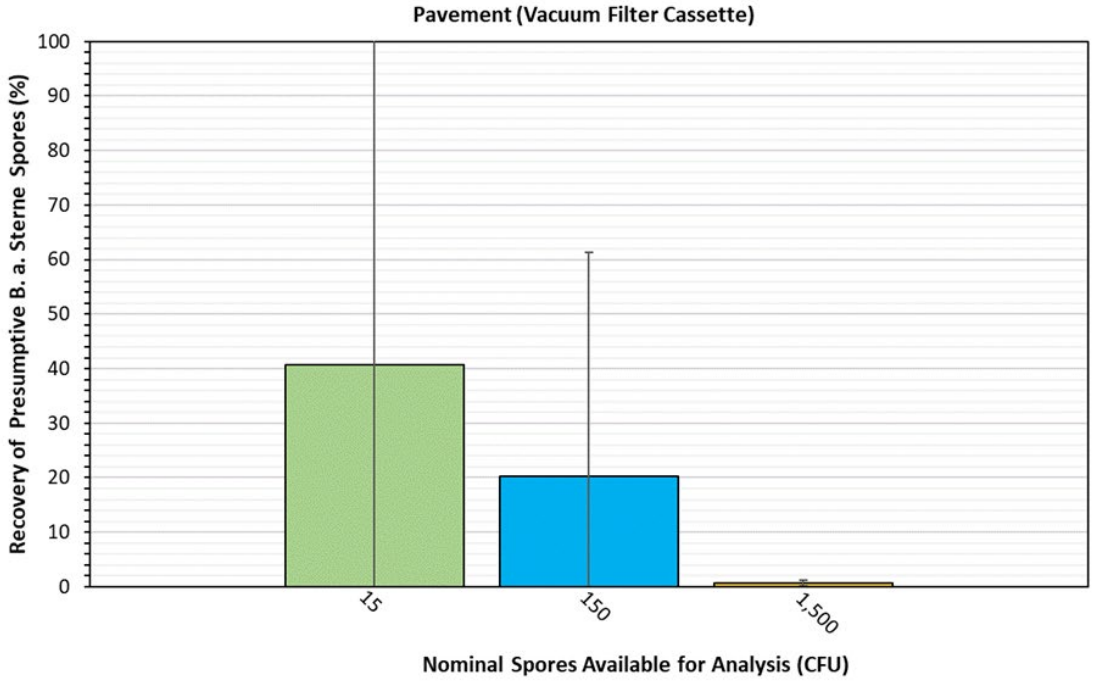

Figure 53. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Pavement Vacuum Filter Cassette Samples Using SBA Medium

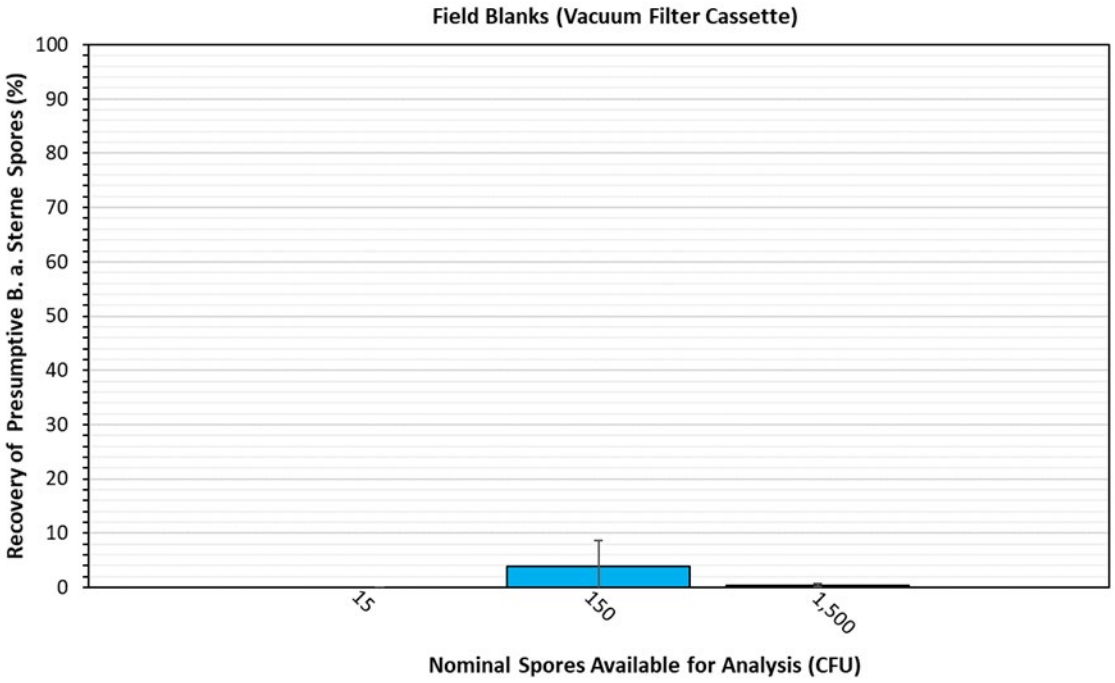

Figure 54. Percent Recovery Efficiencies (Average ± One Standard Deviation of N = 5 Replicates) of Presumptive *B. a.* Sterne Spores from Field Blank Vacuum Filter Cassette Samples Using SBA Medium

A subset of colonies recovered were screened using real-time PCR assays targeting the chromosomal and pXO1 gene targets. A total of 50 colonies isolated from VFCs were screened; of those colonies screened, 68% (34) were confirmed as correctly identified. The 16 presumptive *B. a.* Sterne colonies that were real-time PCR negative artificially inflated the percent recovery values reported for culture and highlights the importance of screening presumptive *B. a.* Sterne colonies following isolation in culture.

A representative, qualitative illustration of the dirt collected on the VFCs is shown in Figure 55 for the various surfaces sampled. There was a range of observable discoloration and/or noticeable particulate matter on the VFCs, especially for the Carpet and Subway Car Filter samples. The images help convey that the analytical methods were applied to very challenging matrices and were far from being applied to pristine samples in a laboratory setting as that associated with method development.

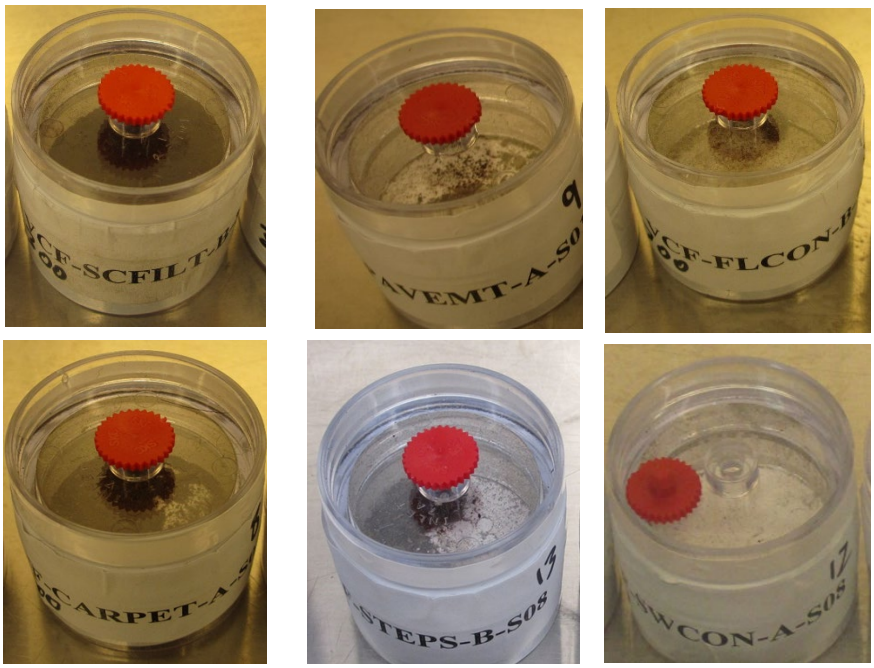

Figure 55. Vacuum Filter Cassettes (Top Left to Right: Subway Car Filter, Pavement, Floor Concrete; Bottom Left to Right: Carpet, Steps, Sidewalk Concrete)

3.2.2 Vacuum Filter Cassette TSB Enrichment

For VFC samples, *B. a.* Sterne morphology was not isolated from turbid TSB broth on SBA streak plates from non-field blank samples. The TSB broth suspension, in all cases, was screened using real-time PCR assays. For the Floor (Concrete), Sidewalk Concrete, Pavement (Asphalt) samples, and field blanks, TSB broth enrichment was PCR positive at a lower spore loading level than RV-PCR positive, indicating that spores were not being physically removed from the filter of the VFC samples. This increased sensitivity of TSB-enriched filters compared to RV-PCR enrichment may be an artifact of liquid inoculation of the filters and may not reflect the natural collection of spores from surfaces using VFCs. Liquid inoculation of the field blank samples, where the spore suspension was applied directly to the filter with no particulates present for spore attachment, had the largest difference in detection limit between TSB enrichment and RV-PCR, where TSB enrichment was two orders of magnitude more sensitive. This result further indicates that the spores tend to adhere to the surface of the inoculated filter during spore recovery steps; however, if spores attached to particulates within the filter cassette, physical recovery improved, as indicated by a closer positive detection limit between TSB enrichment and RV-PCR for samples containing particulates. A summary of the PCR results of TSB-enriched samples is presented in Appendix T.

3.2.3 RV-PCR Method

A summary of the average and sample standard deviation of the RV-PCR ΔCt values for the detection of *B. a.* Sterne spores recovered from VFC samples are presented in Table 7. The ΔCt results are plotted in Figure 56 through Figure 62, with each plot associated with a specific surface. The summary table and associated plots follow the same column header and x-axis labeling convention as used for the presentation of culture results. Most notably, the nominal number of spores available for analysis of 15, 150, and 1,500 CFU are used; it represents the maximum number of spores available, assuming a 100% recovery efficiency and half the sample extract is available for RV-PCR analysis. The average quantity of spores determined available are presented in the summary tables. The 0-spore available condition is included in the plots because meaningful RV-PCR results can be obtained, unlike that for a recovery efficiency. The plots all depict an area shaded in red that is the region of a negative confirmation result and an area of green that is a positive confirmation result, delineated by both the chromosomal and pXO1 gene target ΔCt values having to be ≥ 9 to be a positive result. The RV-PCR results for each VFC sample analyzed are presented in Appendix R.

Table 7. RV-PCR Analyses of Vacuum Filter Cassette Surface Samples for Detection of *B. a.* Sterne Spore Chromosomal and pXO1 Gene Targets (N = 3 Replicates for 0 Nominal Spike; N = 5 for 15, 150, and 1,500 Nominal Spike)

Surface Sample (Surface ID)	Spores Available for Analysis (CFU)		ΔCt (X ± σ)	
	Nominal[a]	Determined[b] (X ± σ)	Chromosomal Gene Target	pXO1 Gene Target
Floor (Concrete) (FLCON)	0	0	0	0
	15	17 ± 5	0.4 ± 0.9	0.6 ± 1.0
	150	170 ± 50	15.6 ± 8.9	15.7 ± 9.0
	1,500	1,700 ± 500	18.4 ± 3.4	18.6 ± 3.3
Steps (Metal) (STEPS)	0	0	0	-0.2 ± 0.3
	15	16 ± 5.6	2.9 ± 5.5	2.9 ± 5.6
	150	160 ± 56	12.6 ± 7.6	12.9 ± 7.1
	1,500	1,600 ± 560	15.9 ± 3.8	16.0 ± 3.8
Carpet (CARPET)	0	0	2.2 ± 3.9	2.2 ± 3.8
	15	16 ± 5.2	4.0 ± 5.6	4.0 ± 5.5
	150	160 ± 52	16.4 ± 2.0	16.0 ± 2.2
	1,500	1,600 ± 520	18.9 ± 3.9	19.0 ±3.9
Subway Car Filter (SCFILT)	0	0	0	0
	15	17 ± 4.8	0	0.1 ± 0.3
	150	170 ± 48	0	2.0 ± 4.4
	1,500	1,700 ± 480	3.4 ± 5.6	4.1 ± 6.0
Sidewalk (Concrete) (SWCON)	0	0	0	0.9 ± 1.6
	15	14 ± 2.3	0.5 ± 1.1	1.0 ± 1.3
	150	140 ± 23	14.8 ± 9.2	14.6 ± 9.3
	1,500	1,400 ± 230	22.2 ± 5.7	22.3 ± 5.7
Pavement (PAVEMT)	0	0	4.9 ± 2.5	5.0 ± 3.4
	15	14 ± 0.9	4.3 ± 6.3	5.3 ± 6.0
	150	140 ± 9.1	4.3 ± 3.4	5.0 ± 3.6
	1,500	1,400 ± 91	13.4 ± 8.2	14.4 ± 6.6
Control Field Blank (FLDBLK)	0	0	3.4 ± 3.9	4.2 ± 4.2
	15	12 ± 2.5	1.0 ± 2.2	1.0 ± 3.1
	150	120 ± 25	0.1 ± 0.3	0.8 ± 2.6
	1,500	1,200 ± 250	20.1 ± 11.3	19.5 ± 11.0

(a) Nominally one-half of the target spore load onto the filter and assuming 100% recovery of spores.
(b) Based on the spiking suspension titer measured each test trial, 100% recovery efficiency, and one-half of extract used for RV-PCR analysis.

In all instances, the ΔCt values for both chromosomal and pXO1 gene targets were < 9 when the VFCs had nominally 15 spores available for analysis, meaning the RV-PCR did not accurately identify the presence of *B. a.* Sterne. It is believed that the poor recovery efficiencies (< 10%) as determined by the culture method and discussed in Section 3.2.1 contributed to the low accuracy of RV-PCR detects rather than interference from the real-world matter collected on the VFCs

during surface sampling. The RV-PCR response for the field blank samples being non-detects for samples with 0, 15, and 150 *B. a.* Sterne spores nominally available supports the idea that poor physical recovery of spores from the VFC affected detection and not due solely to interference from previously collected particulate matter. The RV-PCR did not consistently identify *B. a.* Sterne in the VFC samples until the nominal 1,500 *B. a.* Sterne spores available condition, including the field blank samples.

Consistently, throughout all analyses, very good agreement (ΔCt differed by < 1 between the two gene targets) was obtained for the chromosomal and pXO1 gene targets for all VFC surface samples collected.

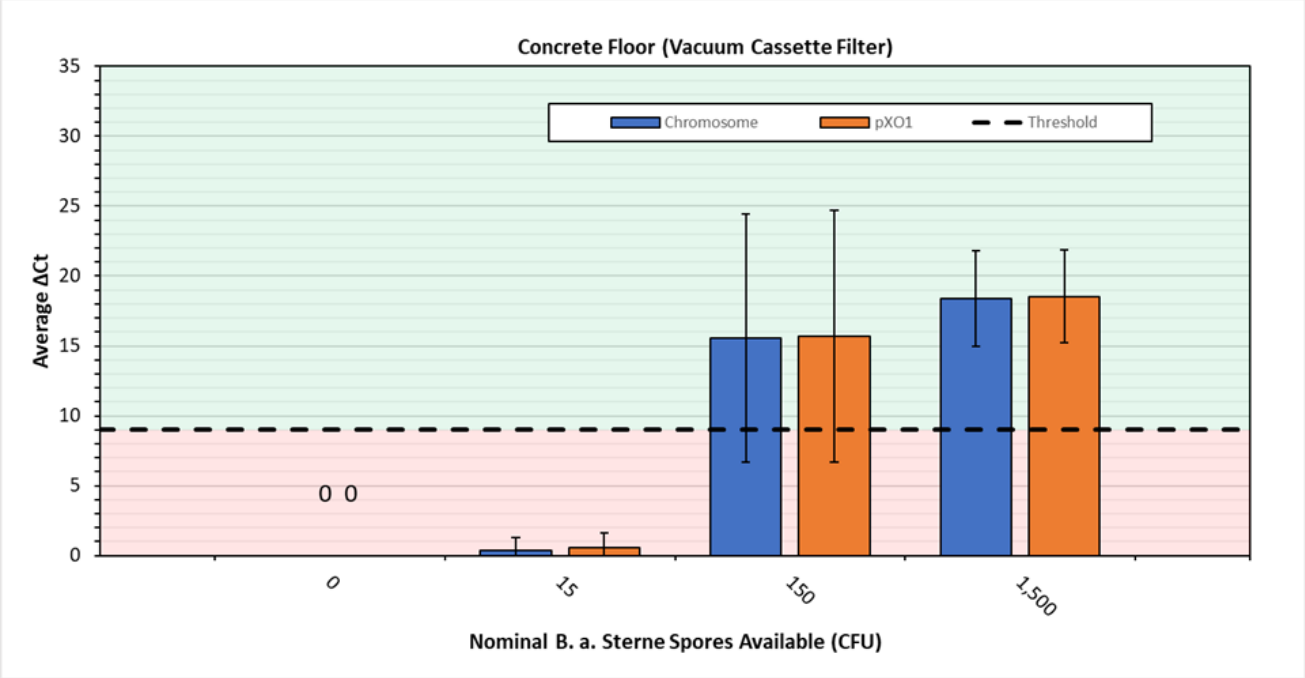

Figure 56. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Concrete Floor Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

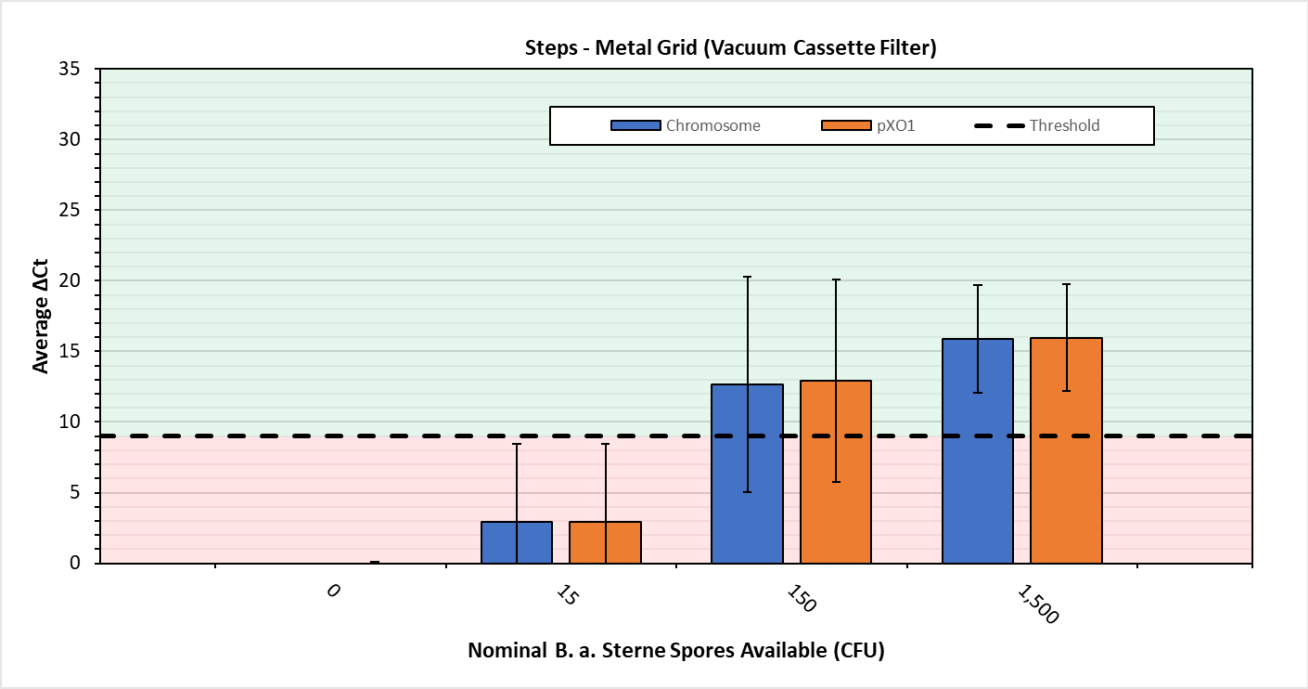

Figure 57. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Steps (Metal) Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

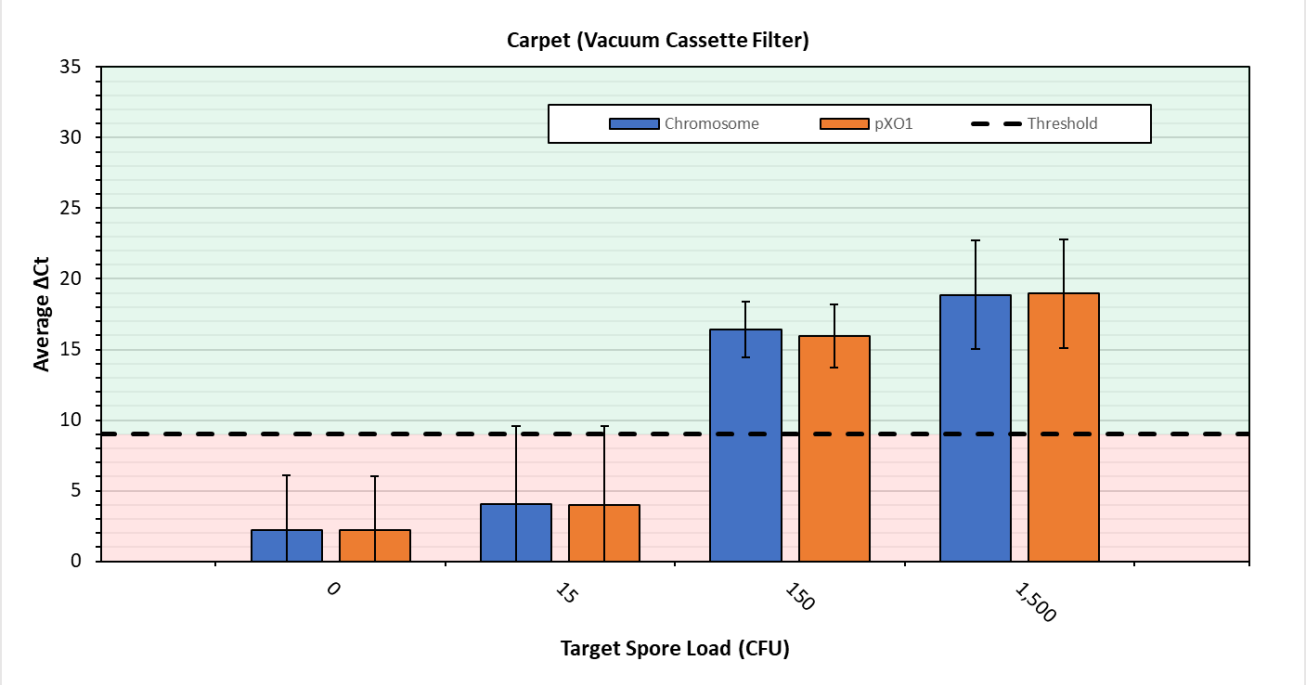

Figure 58. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Carpet Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

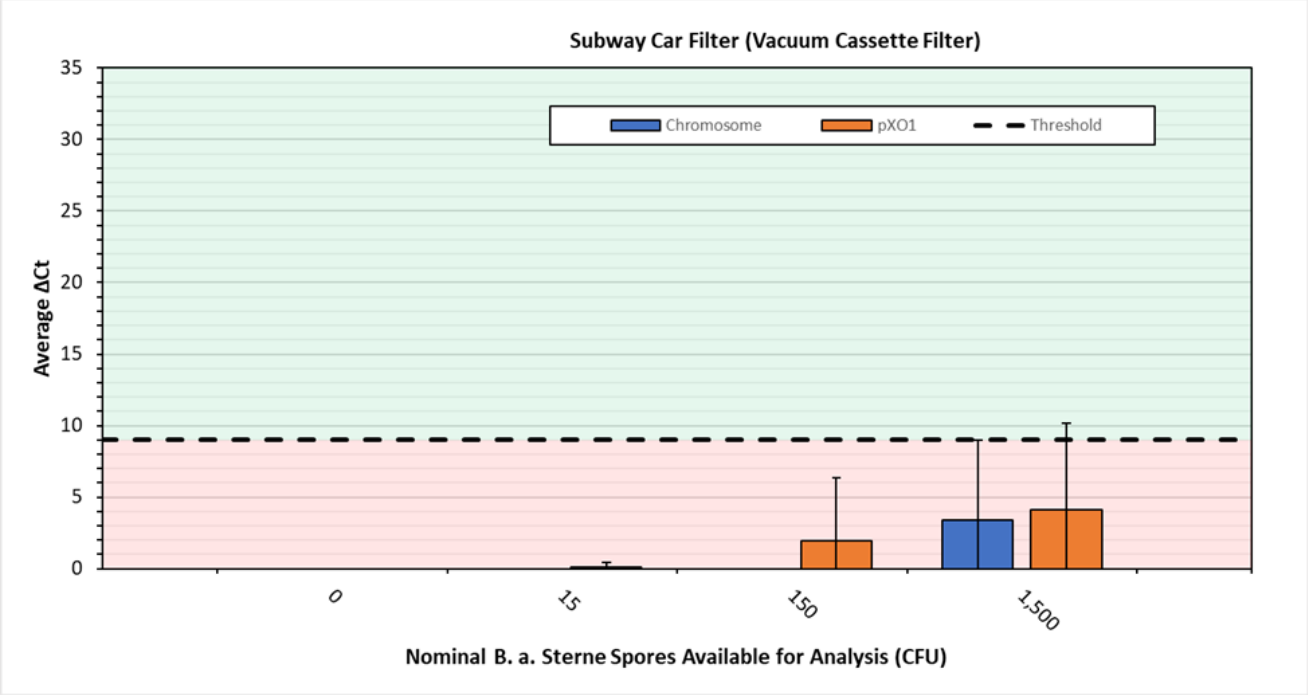

Figure 59. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Subway Car Filter Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

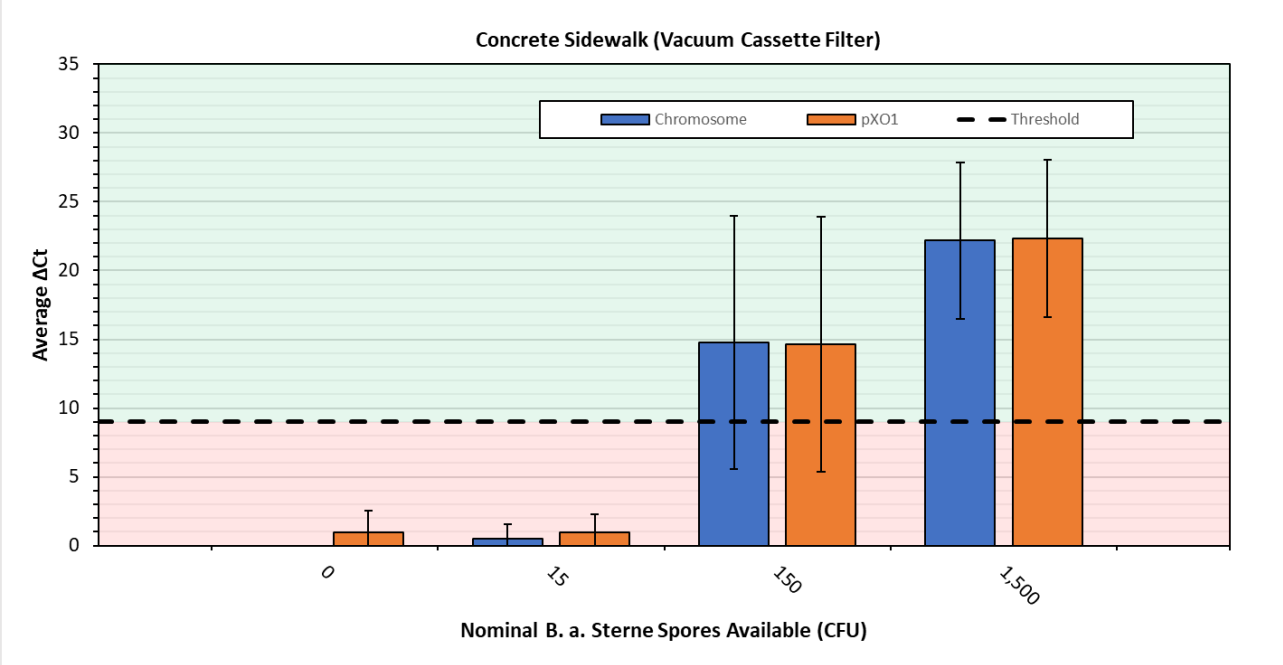

Figure 60. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Concrete Sidewalk Filter Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

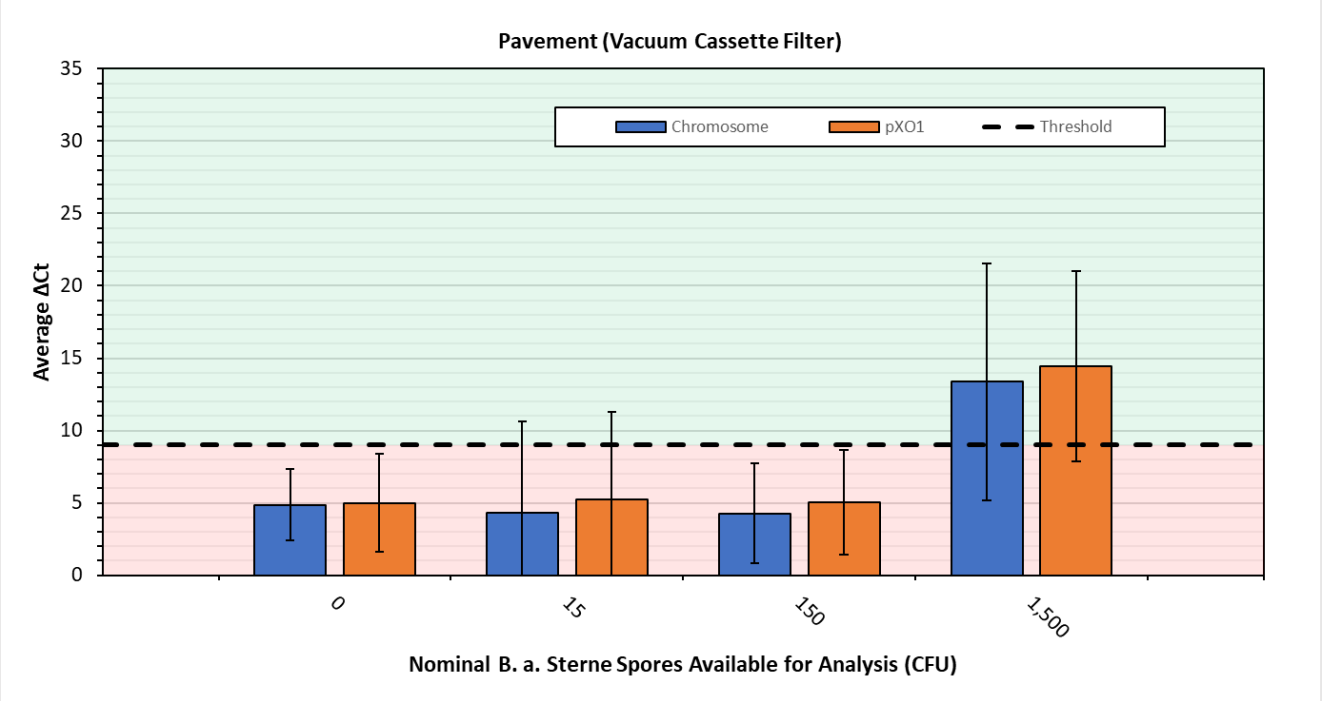

Figure 61. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Pavement Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

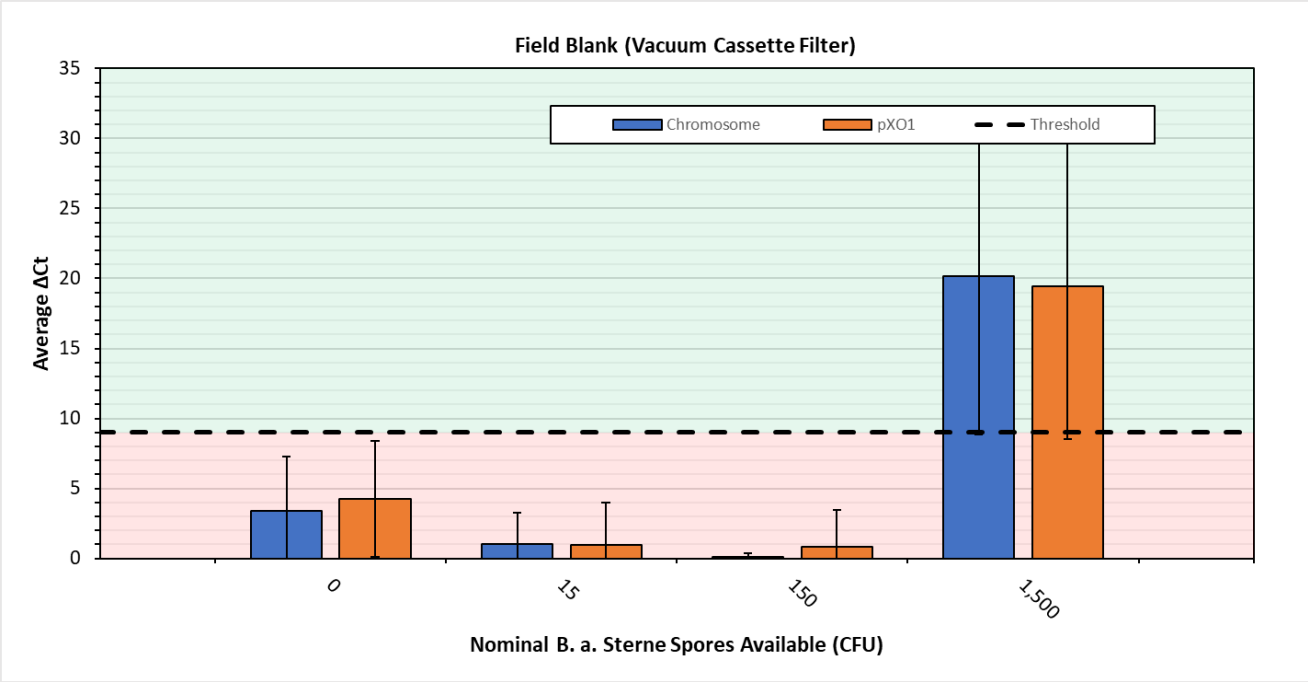

Figure 62. RV-PCR Analysis of *B. a.* Sterne Spores Recovered from Field Blank Vacuum Filter Cassette Samples Using Chromosomal and pXO1 Gene Targets (Average ± One Standard Deviation for N ≥ 3 Replicates); Positive Response Equals ΔCt ≥ 9

3.3 Summary of Detection Accuracy

The results presented in Sections 3.1 and 3.2 can be further reduced to a high-level performance summary of detection accuracy of the two analytical methods and their associated false positive and negative frequencies. For the culture method, false positive was defined as the identification (counting) of one or more presumptive *B. a.* Sterne colonies when none were spiked onto the surface sampler substrate (Sponge-Stick or VFC); false negative was when no presumptive *B. a.* Sterne colonies were counted, yet the sampler substrate was spiked, and an accurate detection when either no colonies were identified in the 0-spike condition or identified for a sampler substrate spiked condition. The positive identification for RV-PCR is as defined in Section 2.4.5 ($\Delta Ct \geq 9$ for both gene targets). A true positive was defined as correctly detecting *B. a.* Sterne in a spiked sample and a true negative as no detection of *B. a.* Sterne in a sampler substrate that was not spiked. A summary of those results is presented in Table 8 and Table 9 for the Sponge-Stick and VFC samples, respectively for each sampled surface.

The RV-PCR analytical method was successful in accurately identifying (97% correct true detection and 100% correct true negative response) the presence or absence of *B. a.* Sterne in the Sponge-Stick samples, meaning the particulate matter previously collected on the Sponge-Stick had minimal adverse impact on method performance. Combining true positives and true negatives, the RV-PCR method had an accuracy of 97.3%. There were no false positives for RV-PCR analyses of Sponge-Stick samples, indicating no cross-contamination. The false negatives (3.2%) for RV-PCR of Sponge-Stick samples were believed to be due, in part, to poor physical recovery of organisms from the sampler substrate as well as likely some loss in sensitivity due to the ambient particulate matter recovered along with the *B. a.* Sterne. The RV-PCR method was able to accurately identify the presence of *B. a.* Sterne in 47% of the VFC samples that were spiked with *B. a.* Sterne spores, but was 100% accurate in identifying true negatives. The relatively low accuracy of RV-PCR to identify the presence of *B. a.* Sterne from VFC samples compared to the Sponge-Sticks is attributed to the low (< 10%) recovery efficiency of *B. a.* Sterne from the VFC substrate.

The culture method's ability to accurately identify *B. a.* Sterne was hampered by the presence of particulate matter previously collected on the Sponge-Stick samples (77% correct true positives). The true negatives were correctly identified in 96% of the samples. Combining true positives and

true negatives, the culture method using SBA medium had an accuracy of 80%. The false negative detections for culture were associated predominantly with samples having a background flora of competing organisms did not permit identification of any colonies with *B. a.* Sterne morphology. The false positives for culture were attributed, and in some instances confirmed with PCR analysis of selected colonies, to presumptive *B. a.* Sterne colonies not being correct, as discussed earlier. The culture method was able to accurately identify the presence of *B. a.* Sterne in 54% of the VFC samples that were spiked with *B. a.* Sterne spores, but was 90% accurate in identifying true negatives. The relatively low accuracy of the culture method to identify the presence of *B. a.* Sterne from VFC samples compared to the Sponge-Sticks is attributed to the low (< 10%) recovery efficiency of *B. a.* Sterne from the VFC substrate.

Table 8. Summary of the Accuracy of the Analytical Method Response to Detect *B. a.* Sterne on Sponge-Stick Samples

Surface ID[a]	Total Number of Samples			SBA Culture / RV-PCR			
	Spiked	Not Spiked	Total	True Positive[b]	True Negative[c]	False Positive[d]	False Negative[e]
FLTILE	15	3	18	12 / 15	3 / 3	0 / 0	3 / 0
				80% / 100%	100% / 100%	0% / 0%	20% / 0%
FLCON	15	3	18	13 / 15	3 / 3	0 / 0	2 / 0
				87% / 100%	100% / 100%	0% / 0%	13% / 0%
STEPS	15	3	18	7 / 15	3 / 3	0 / 0	8 / 0
				47% / 100%	100% / 100%	0% / 0%	53% / 0%
WLTILE	15	3	18	14 / 14	3 / 3	0 / 0	1 / 1
				93% / 93%	100% / 100%	0% / 0%	6.7% / 6.7%
GLSWIN	15	3	18	15 / 15	3 / 3	0 / 0	0 / 0
				100% / 100%	100% / 100%	0% / 0%	0% / 0%
EDPAN(B)	15	3	18	13 / 15	3 / 3	0 / 0	2 / 0
				87% / 100%	100% / 100%	0% / 0%	13% / 0%
GLSPAN	15	3	18	15 / 14	3 / 3	0 / 0	0 / 1
				100% / 93%	100% / 100%	0% / 0%	0% / 6.7%
FLLFIX	15	3	18	14 / 15	3 / 3	0 / 0	1 / 0
				93% / 100%	100% / 100%	0% / 0%	6.7% / 0%
OHSIGN	15	3	18	15 / 15	3 / 3	0 / 0	0 / 0
				100% / 100%	100% / 100%	0% / 0%	0% / 0%
MCMACH	15	3	18	10 / 15	3 / 3	0 / 0	5 / 0
				67% / 100%	100% / 100%	0% / 0%	33% / 0%
SCGRIL	15	3	18	11 / 14	3 / 3	0 / 0	4 / 1
				73% / 93%	100% / 100%	0% / 0%	27% / 6.7%
FLDBLK	15	3	18	15 / 15	3 / 3	0 / 0	0 / 0
				100% / 100%	100% / 100%	0% / 0%	0% / 0%
SWCON	15	3	18	13 / 15	2 / 3	1 / 0	2 / 0
				87% / 100%	66% / 100%	33% / 0%	13% / 0%
EDPAN(A)	15	3	18	13 / 15	3 / 3	0 / 0	2 / 0
				87% / 100%	100% / 100%	0% / 0%	13% / 0%
CWSIGN	15	3	18	11 / 15	3 / 3	0 / 0	4 / 0
				73% / 100%	100% / 100%	0% / 0%	27% / 0%
TELEBO	15	3	18	12 / 15	2 / 3	1 / 0	3 / 0
				80% / 100%	66% / 100%	0% / 0%	20% / 0%
STGRAT	15	3	18	0 / 12	3 / 3	0 / 0	15 / 3
				0% / 80%	100% / 100%	0% / 0%	100% / 20%

Table 8. Summary of the Accuracy of the Analytical Method Response to Detect *B. a. Sterne* on Sponge-Stick Samples (Cont.)

Surface ID	Total Number of Samples			SBA Culture / RV-PCR			
	Spiked	Not Spiked	Total	True Positive[(a)]	True Negative[(b)]	False Positive[(c)]	False Negative[(d)]
CWPNTD	15	3	18	5 / 13	3 / 3	0 / 0	10 / 2
				33% / 87%	100% / 100%	0% / 0%	67% / 13%
GRNBEN	15	3	18	12 / 14	3 / 3	0 / 0	3 / 1
				80% / 93%	100% / 100%	0% / 0%	20% / 6.7%
SUM	285	57	342	220 / 276	55 / 57	2 / 0	65 / 9
Percent				77% / 97%	96% / 100%	3.4% / 0%	23% / 3.2%

(a) Surface ID codes defined in Table 1

(b) Number of positive responses (for each analytical method) to filters that were spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of spiked samples.

(c) Number of negative responses (for each analytical method) to filters that were not spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of samples not spiked.

(d) Number of positive responses (for each analytical method) to filters that were not spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of samples not spiked.

(e) Number of negative responses (for each analytical method) to filters that were spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of spiked samples.

Table 9. Summary of the Accuracy of the Analytical Method Response to Detect *B. a.* Sterne on Vacuum Filter Cassette Samples

Surface ID	Total Number of Samples			SBA Culture / RV-PCR			
	Spiked	Not Spiked	Total	True Positive[(a)]	True Negative[(b)]	False Positive[(c)]	False Negative[(d)]
FLCON	15[(a)]	3	18	11 / 8	2 / 3	1 / 0	4 / 6
				73% / 53%	67% / 100%	33% / 0%	27% / 40%
STEPS	15	3	18	8 / 10	3 / 3	0 / 0	7 / 5
				53% / 67%	100% / 100%	0% / 0%	47% / 33%
CARPET	15	3	18	7 / 12	2 / 3	1 / 0	8 / 3
				47% / 80%	67% / 100%	33% / 0%	53% / 20%
SCFILT	15	3	18	7 / 1	3 / 3	0 / 0	8 / 14
				47% / 6.7%	100% / 100%	0% / 0%	53% / 93%
SWCON	15	3	18	9 / 9	3 / 3	0 / 0	6 / 6
				60% / 60%	100% / 100%	0% / 0%	40% / 40%
PAVEMT	15	3	18	8 / 5	3 / 3	0 / 0	7 / 10
				53% / 33%	100% / 100%	0% / 0%	47% / 67%
FLDBLK	15	3	18	7 / 4	3 / 3	0 / 0	8 / 11
				47% / 27%	100% / 100%	0% / 0%	53% / 73%
SUM	105	21	126	57 / 49	19 / 21	2 / 0	48 / 55
Percent				54% / 47%	90% / 100%	10% / 0%	46% / 52%

(a) Number of positive responses (for each analytical method) to filters that were spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of spiked samples.
(b) Number of negative responses (for each analytical method) to filters that were not spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of samples not spiked.
(c) Number of positive responses (for each analytical method) to filters that were not spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of samples not spiked.
(d) Number of negative responses (for each analytical method) to filters that were spiked with *B. a.* Sterne; percentage calculated by dividing number of occurrences by number of spiked samples.
(e) One sample was lost due to filter vial leak.

Table 10 provides a similar summary of method response comparison, but as a measure of the consistency of both methods yielding the same response of whether *B. a.* Sterne was detected, or not, for the Sponge-Stick and VFC surface samples. The methods agreed (both giving the same response to a correct positive identification for the presence of *B. a.* Sterne) in 77% of the Sponge-Stick samples analyzed and to a correct negative response 96% of the time samples were absent of *B. a.* Sterne. As noted in Table 10, there were 62 instances when both analytical methods yielded a negative response, which was due to 7 *B. a.* Sterne spiked Sponge-Stick samples having a negative response for both analytical methods. Otherwise, 55 of the 57 0-spike samples resulted in a negative response by both analytical methods. The discrepancy in the two

methods was attributed to interference in the culture method caused by the particulate matter previously collected on the Sponge-Sticks. The methods agreed only 40% of the time to the presence of *B. a.* Sterne spores in the VFC samples. As discussed earlier in this section, both methods had performed poorly due to poor spore recovery more so than a result of interference caused by the collected particulate matter on the VFC. As noted in Table 10, there were 54 instances when both analytical methods yielded a negative response to the presence of *B. a.* Sterne, yet there were only 21 0-spike samples that should have been absent of *B. a.* Sterne.

The last two columns in Table 10 indicate the number of occurrences and percentage of occurrences when the two analytical methods yielded different outcomes regarding the detection of *B. a.* Sterne. Combining the results of the last two columns, the two methods did not agree in 18% of the Sponge-Stick samples and 28% of the VFC samples. The poor *B. a.* Sterne spore recovery efficiency from the VFC and presence of flora/grime in other samples were believed to be the main contributors to the analytical response discrepancy.

Table 10. Paired Overall Positive and Negative *B. a.* Sterne Detection Results and Frequency for Culture and Molecular Analysis Methods, Sponge-Stick and Vacuum Filter Cassette Sample Results Pooled

Sample Type	Total Number of Samples			SBA Culture and RV-PCR			
	Spiked	Not Spiked	Total	Pos/Pos[a]	Neg/Neg[b]	Pos/Neg[c]	Neg/Pos[d]
Sponge-Sticks	285	57	342	218	62[e]	4	58
				77%	96%[e]	3.2%	19%
VFC	105	21	126	36	54[f]	22	13
				40%	90%[f]	18%	10%

(a) Both the culture and molecular responses positively identified the presence of *B. a.* Sterne correctly in samples that were spiked; percentage calculated by dividing number of occurrences by number of spiked samples.
(b) Both the culture and molecular responses correctly gave a negative response to the presence of *B. a.* Sterne correctly in samples that were not spiked; percentage calculated by dividing number of occurrences by number of spiked samples.
(c) Culture yielded a positive response for *B. a.* Sterne and molecular response was negative for the presence of *B. a.* Sterne; percentage calculated by dividing number of occurrences by the number of samples analyzed.
(d) Culture yielded a negative response for *B. a.* Sterne and molecular response was positive for the presence of *B. a.* Sterne; percentage calculated by dividing number of occurrences by the number of samples analyzed.
(e) 55 of the 57 samples not spiked with *B. a.* Sterne resulted in both RV-PCR and culture both having a negative response, and the percentage given is based on those responses to the 57 0-spiked samples. The 62 Neg/Neg responses are due to 7 spiked samples yielding a negative response for both analytical methods.
(f) 19 of the 21 samples not spiked with *B. a.* Sterne resulted in both RV-PCR and culture both having a negative response, and the percentage given is based on those responses to the 21 0-spiked samples. The 54 Neg/Neg responses are due to 35 spiked samples yielding a negative response for both analytical methods.

4.0 QUALITY ASSURANCE/QUALITY CONTROL

Quality assurance (QA)/quality control (QC) procedures were performed in accordance with the Testing and Evaluation (T&E II) Program Quality Management Plan (QMP), Version 1 and the TO 09 Quality Assurance Project Plan (QAPP) (Battelle, 2017). The QA/QC procedures and results are summarized below.

4.1 Equipment Calibration

All equipment (e.g., pipettes, incubators, water baths, refrigerators/freezers) used at the time of the evaluation were verified as being certified, calibrated, or validated.

4.2 QC Results

QC efforts conducted during RWI testing included positive and negative controls for both spread plate samples and qPCR. In addition, they included quantification of both the spore bank and *B. a.* Sterne spike suspensions to verify either CFU/mL titer or target spike concentrations.

Positive controls (PC) and NTCs were included for each RV-PCR assay and in all cases the 50-pg PC consistently resulted in Ct values in the mid-20s, as expected. There were two instances that NTC wells generated a fluorescence signal (Ct of 44.5 and 43.5) that crossed the threshold, occurring during Sponge-Stick Trial 12 on September 10, 2018 and VFC Trial 7 on January 28, 2019. The signal was detected in the pXO1 assay, not the chromosome assay, and all other NTC wells, zero spike samples, and T_0 samples did not cross the threshold, indicating that this erroneous signal did not have an impact on the Ct values generated for samples. Applied Biosystems 7500 Fast system performance was assessed according to internal standard operating procedures (SOPs) and maintained at regular intervals—monthly (optical and background calibration), every 6 months (dye calibration), and annually (RNase P calibration). For culture, the PC spore stock maintained a single morphological appearance consistent with *B. a.* Sterne throughout the study, as determined at the beginning of each trial. Media and reagents used for culture analysis were screened (negative controls) and had no growth, showing that reagents used were not the source of contamination. The field blank samples provided a means to baseline the method performance without competing flora and grime.

4.3 Operational Parameters

Micropipettes, thermometers, and timers used were calibrated against a traceable standard at regular intervals (every 6 months or annually) and used only within acceptable calibration interval established by internal SOPs.

4.4 Audits

4.4.1 Performance Evaluation Audit

Performance evaluation (PE) audits were conducted to assess the quality of the results obtained during these experiments. Table 11 summarizes the PE audits that were performed; equipment was within acceptable tolerance range.

Table 11. Performance Evaluation Audits

Measurement	Audit Procedure	Allowable Tolerance	Actual Tolerance
Volume of liquid from micropipettes	Gravimetric evaluation	± 10%	Passed calibration as found/as returned
Time	Compared to independent clock	± 2 sec/hour	Passed calibration as found/as returned
Temperature	Compared to independent calibrated thermometer	± 2°C	Passed calibration as found/as returned

4.4.2 Technical Systems Audit

Observations and findings from the technical system audit (TSA) were documented and submitted to the laboratory technical lead for response. TSA was conducted on June 15, 2018 to ensure that tests were being conducted in accordance with the appropriate QAPP and QMP. As part of the audit, test procedures were compared to those specified in the QAPP and work instructions, and data acquisition and handling procedures were reviewed. None of the findings of the TSA required corrective action.

4.4.3 Data Quality Audit

At least 10% of data acquired during the evaluation were audited. Data were reviewed in December 2018 and January 2019. A QA auditor traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were verified. Only minor issues were noted with the data, mostly data transcription errors that were corrected.

4.5 QA/QC Reporting

Each assessment and audit was documented in accordance with the QAPP and QMP. For these tests, findings were noted (none were significant) in the data quality audit, and no follow-up corrective action was necessary. The findings were mostly minor data transcription errors requiring some recalculation of efficacy results, but none were gross errors in recording. QA/QC procedures were performed in accordance with the QAPP.

4.6 Data Review

Records and data generated in the evaluation received a QC/technical review before they were utilized in calculating or evaluating results and prior to incorporation in this report.

5.0 SUMMARY OF METHOD OBSERVATIONS AND EXPERIENCES

While implementing the method, key observations and experiences were noted that will be useful to understand and/or take into consideration for future iterations or versions of the method. Key observations were:

- The RV-PCR method requires great care and diligence to implement effectively. Most notable, the method required changing gloves between procedural samples for each step, which is time-consuming. Glove changing is critical to avoid cross-contamination samples, which would negatively impact key decisions in the response, response timelines, credibility, and cost.
- During the RV-PCR method, when applying vacuum to the filter vial manifold, the filtrate pooled in the manifold reservoir and contacted the bottom of the filter vials near the vacuum source. It is recommended to increase the depth of the bottom section of the manifold so that the filtrate does not pool and contact the bottom of filter vials.
- The RV-PCR sample analyses were performed in batches of 16 per trial using a single system based on initial trials to implement the methods. The 16 samples were the maximum that was deemed reasonable to process considering a normal 8:00 AM to 5:00 PM workday, without overtime and/or a night shift that may be used by the ERLN if actual samples were being processed. A single trial was completed over 4 consecutive days of operation, starting with sample spiking on Day 1. (Had these been actual filters collected post-biological release, the spiking activity would, obviously, not be performed by the ERLN.)
- A 16-hour incubation duration for RV-PCR was used in this study, but the EPA method typically uses a 9-hour incubation duration. It is reasonable to initially use the 9-hour incubation because the RV-PCR ΔCt was commonly over 15 for the air quality and non-air quality filters analyzed if the filters had 150 or more spores available. In practice, longer incubation times could be implemented for selected samples to confirm a negative response with a 9-hour incubation time.
- Estimated staff time to process 16 samples was approximately 64 hours and $1,500 of consumables. The 64 hours of staff time budget was approximately distributed by:
    - 8 hours for activities related specifically to the spiking of the filters being assessed, which was a requirement of the study, but not an activity that would be performed had these been actual field samples. This task included time to prepare the stock suspensions, enumerate stock suspension, spike the filters, and associated documentation.
    - 10 hours for spore recovery.
    - 10 hours for culture analysis.
    - 24 hours for RV-PCR analysis.
    - Additionally, 4 hours was needed for PCR confirmation analysis of eight samples, when performed.

- Had the EPA 2012 method been followed without any changes (most notably the samples would not be split for analysis and either the culture only or the RV-PCR method only been used), a batch of 16 samples would take an estimated 34 labor hours and $1,000 in materials to perform culture analysis (with PCR confirmation of at least three colonies per sample). To process the same number of samples, an estimated 40 hours and $1,200 would be required using RV-PCR analysis. Each of the analytical methods would take 2 or 3 days:
    - The benefit to RV-PCR is that *B. a.* Sterne can be detected in sample matrices with high amounts of background flora and grime. For culture analysis, the growth of viable *B. a.* Sterne spores may be masked by background flora and grime in environmental samples, and therefore go undetected.
- The extract suspension from Sponge-Sticks tended to clog the RV-PCR filter vials. As a result, the below two rules were applied to expedite sample processing and the inclusion of buffer washes:
    - At 15-minute, post-sample addition to the filter vial. If the sample did not pass through the filter vial, a reduced volume of high and low salt wash buffer (5 mL) was added, rather than omitting one or both entirely.
    - At 1-hour, post-sample addition to the filter vial. If the sample did not pass through the filter vial, the high and low salt wash steps were omitted.
- For Sponge-Sticks, colony PCR screening results indicate that the microbiologist's identification of colonies were correct 93% of the time, from a total of 229 colonies screened. There was one instance where the presumptive ID of a colony was negative and colony PCR was positive, and 15 instances where presumptive *B. a.* Sterne colonies were colony PCR negative.
- For VFCs, colony PCR screening results indicate that the microbiologist's identification of colonies were correct 68% of the time, from a total of 50 colonies screened. There were 16 instances where presumptive *B. a.* Sterne colonies were colony PCR negative.
    - Seventeen (17) colonies that were streaked and isolated from the TSB enrichment broth were screened using colony PCR. Only three were positive and each of the three colonies was isolated from field blank samples that did not have background flora competition.
- For Sponge-Sticks, TSB enrichment did not lead to the isolation of *B. a.* Sterne on SBA streak plates. Real-time PCR analysis of the turbid TSB resulted in three positive samples from Street Grating surface, although the RV-PCR enrichment Ct values were lower, indicating that spore recovery method for Sponge-Sticks and RV-PCR enrichment was more sensitive than TSB enrichment of the extracted sponge. The enrichment analysis was included for the last four trials of Sponge-Sticks.
- Data suggest that *B. a.* Sterne spiked onto VFCs are not efficiently removed from the filter. As a result, TSB enrichment, which is an enrichment of the VFC filter following spore recovery, is PCR positive at a lower loading level than RV-PCR, which is an enrichment of the spore recovery suspension:

  - Recommend evaluating recovery method and perhaps incorporating a vortex step in addition to the bath sonication of the VFC.
  - Vacuum (VFC) field blank samples containing 0, 30, and 300 spores had no observed growth (turbidity) in filter vials following overnight incubation in BHIB (RV-PCR T_{final} enrichment) and resulted in RV-PCR negative samples. The extracted filter from these field blank samples that was enriched with TSB did grow during overnight incubation and resulted in TSB enrichment PCR positive samples.

- For Sponge-Sticks and VFCs, *B. a.* Sterne was not isolated from streaking turbid TSB onto SBA plates from non-field blank samples. However, *B. a.* Sterne was present in the TSB enrichment broth for many of the VFC samples, as determined by PCR analysis of an aliquot of the broth.

6.0 CONCLUSIONS AND RECOMMENDATIONS

The foremost conclusion is that the RV-PCR *B. anthracis* analysis method was > 97% accurate in correctly identifying the presence or absence of *B. a.* Sterne in Sponge-Stick samples that had previously collected background material from real-world surface sampling. The culture method was less accurate (77%) in correctly identifying the presence or absence of *B. a.* Sterne in the same Sponge-Stick samples, meaning the presence of real-world material collected during surface sampling can hinder the culture method performance. The 18 field blank samples analyzed – 15 samples spiked with *B. a.* Sterne and 3 samples not spiked – were 100% accurately identified by the culture method.

Neither the culture nor molecular analytical methods performed as well with surface samples collected using the VFCs compared to the Sponge-Stick sampling method. The decrease in performance as measured by the accuracy of properly identifying the presence or absence of *B. a.* Sterne spiked onto the samples was attributed primarily to poor physical recovery of *B. a.* Sterne from the VFC MCE collection substrate and, also, possibly, to the collected ambient particulate matter. Physical recovery of spores could potentially be improved by modification of the method; the addition of a vortex step after sonication of the VFC MCE collection substrate is recommended to be assessed. It is possible that the spiking method (drops of a *B. a.* Sterne suspension applied directly onto the VFC collection substrate) affected the physical recovery of organisms for subsequent analysis. Collection of the *B. a.* Sterne as an aerosol while simultaneously collecting the ambient particulate matter (as would be the case in an actual sampling campaign following a bioterrorism incident) may yield improved recovery efficiencies. This effect, however, may be most apparent when there is an opportunity to collect much ambient particulate matter.

One recommendation is therefore to assess the impact that spiking of *B. a.* Sterne spores onto the VFC substrates has on the recovery and subsequent analyses. The liquid suspension spiking method may bias the recovery efficiencies favorably (higher efficiency) or unfavorably. Specifically, it is recommended to expand the study by depositing an aerosol of *B. a.* Sterne onto real-world “dirty” surfaces, and then sampling the comingled background material with deposited *B. a.* Sterne using the VFC. It is also recommended to perform the same sampling effort using the Sponge-Stick sampling method for comparison and completeness. RV-PCR can

be used to positively identify viable *B. a.* Sterne in presence of complex, dirty sample matrices from Sponge-Stick surface samples. The background flora and grime collected on the Sponge-Sticks can reduce the lower limit of detection and/or suppress the sensitivity of the *B. a.* Sterne signal, but samples with as few as a nominal quantity of 15 *B. a.* Sterne spores could routinely be positively identified.

The results from this study will be useful to those analyzing samples collected following a bioterrorism incident by having an understanding that the culture results may be confounded by an overwhelming presence of background flora, obscuring the presence of *B. anthracis* spores.

7.0 REFERENCES

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EVALUATION OF ANALYTICAL METHODS FOR THE DETECTION OF *BACILLUS ANTHRACIS* SPORES: COMPATIBILITY WITH REAL-WORLD SAMPLES COLLECTED FROM OUTDOOR AND SUBWAY SURFACES

APPENDICES A-T

APPENDIX A: TARGET SURFACES

Figure A-1. Carpet Surface Located by the Jackie O Entrance to Station, Off 42nd Street – Sampled with Vacuum Filter Cassette

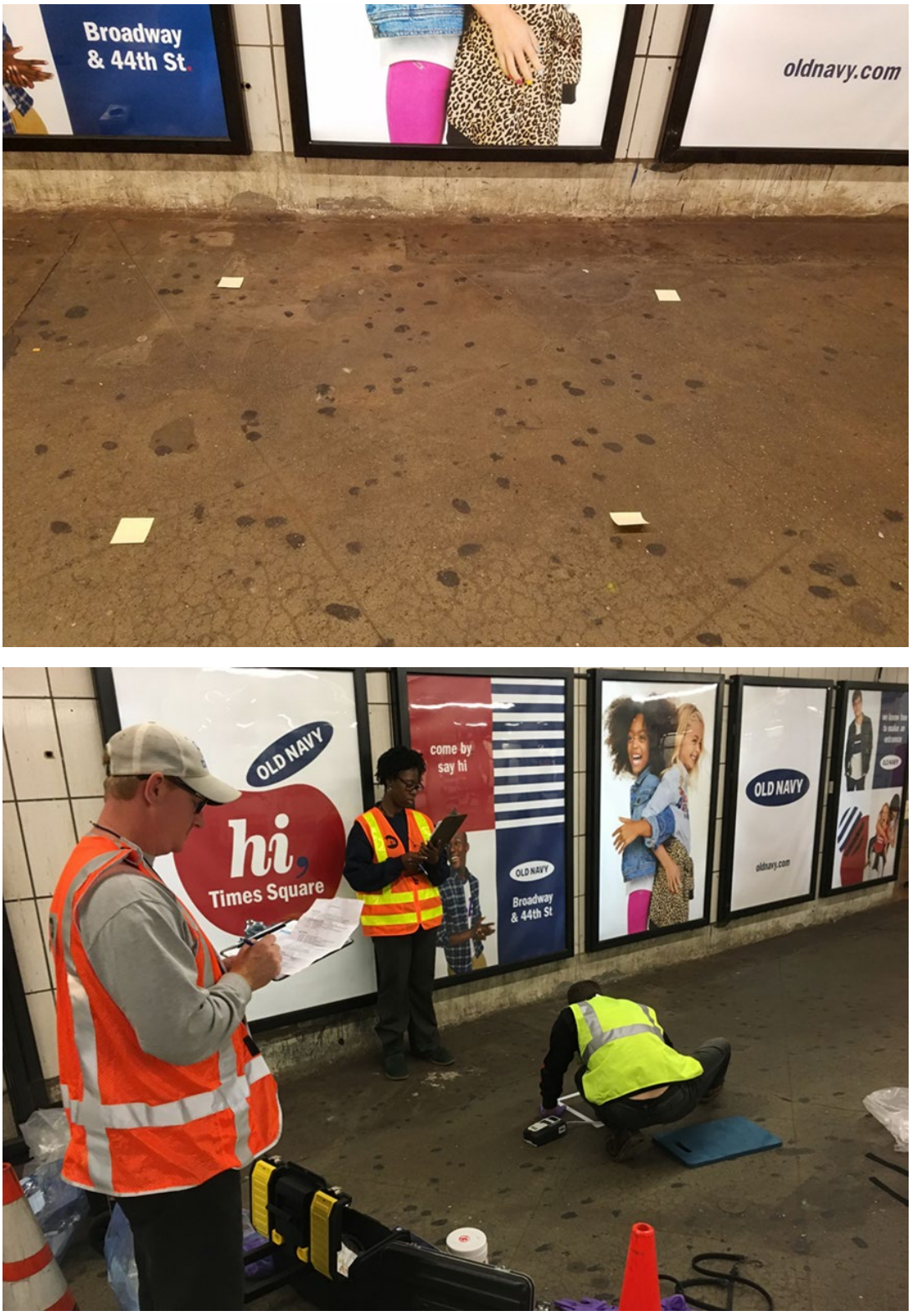


Figure A-2. Concrete Floor Located Just Below Times Square Police Station, One Level Down – Sampled with Sponge-Sticks and Vacuum Filter Cassette

Figure A-3. Crosswalk Signals Located Above Various Intersections on Broadway, 44th, and 7th Street – Sampled with Sponge-Sticks

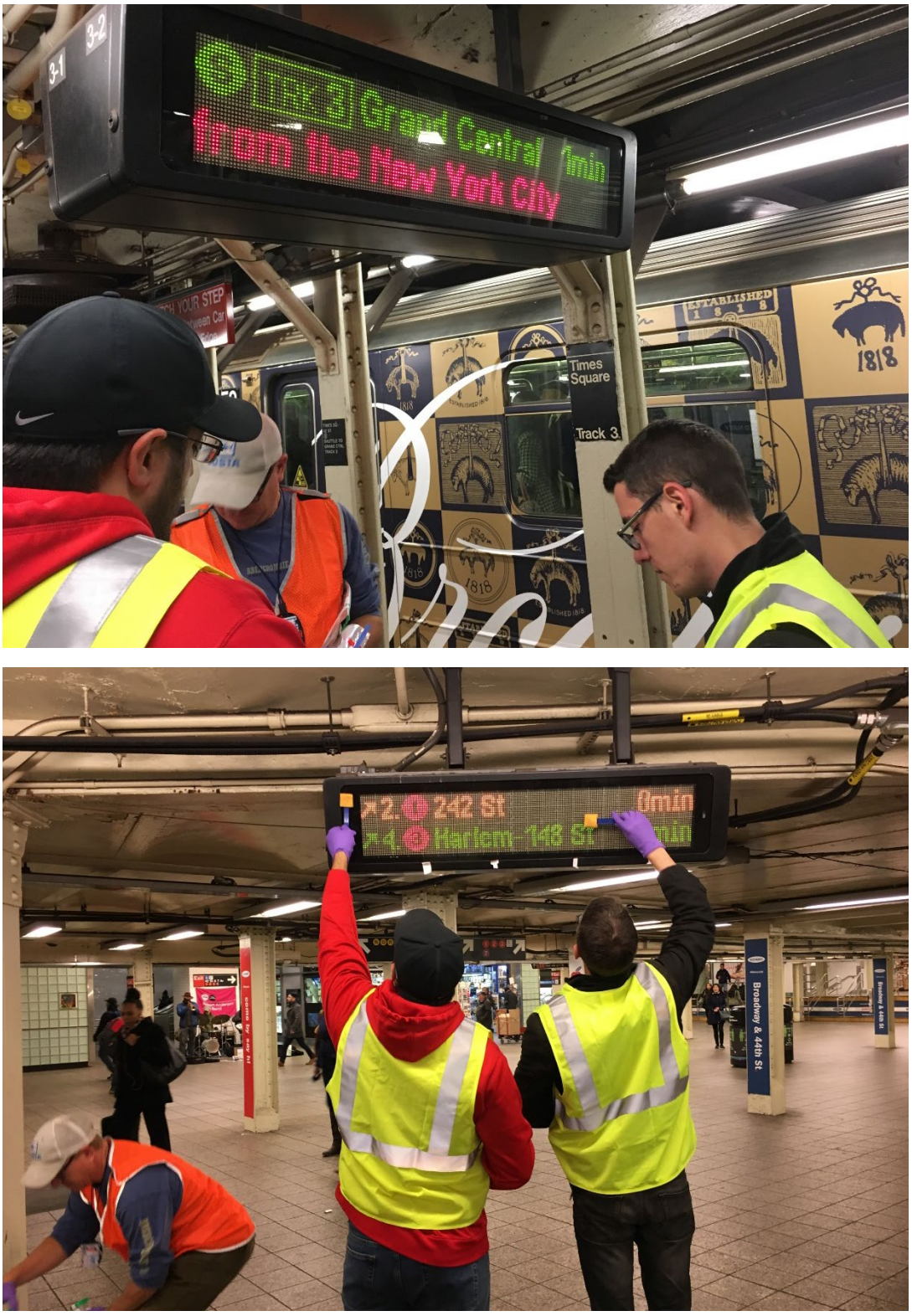


Figure A-4. Electronic Display Panels (Below Ground) Located in Times Square 42nd Street Station, Near Track 3 – Sampled with Sponge-Sticks

Figure A-5. Electronic Display Panels (Above Ground) Located Above Subway Entrance Next to the Times Square Police Station – Sampled with Sponge-Sticks

Figure A-6. Floor Tiles Located at the Grand Central Shuttle Landing, Between Tracks 1 and 3, Near the Control Booth – Sampled with Sponge-Sticks

Figure A-7. Fluorescent Light Fixtures Located Near the PSU by the Record Mart – Sampled with Sponge-Sticks

Figure A-8. Glass Panels on Subway Map Displays Located Near Madison and 42nd Street Subway Exit – Sampled with Sponge-Sticks

Figure A-9. Glass Windows/Facades Located Near the PSU by the Record Mart – Sampled with Sponge-Sticks

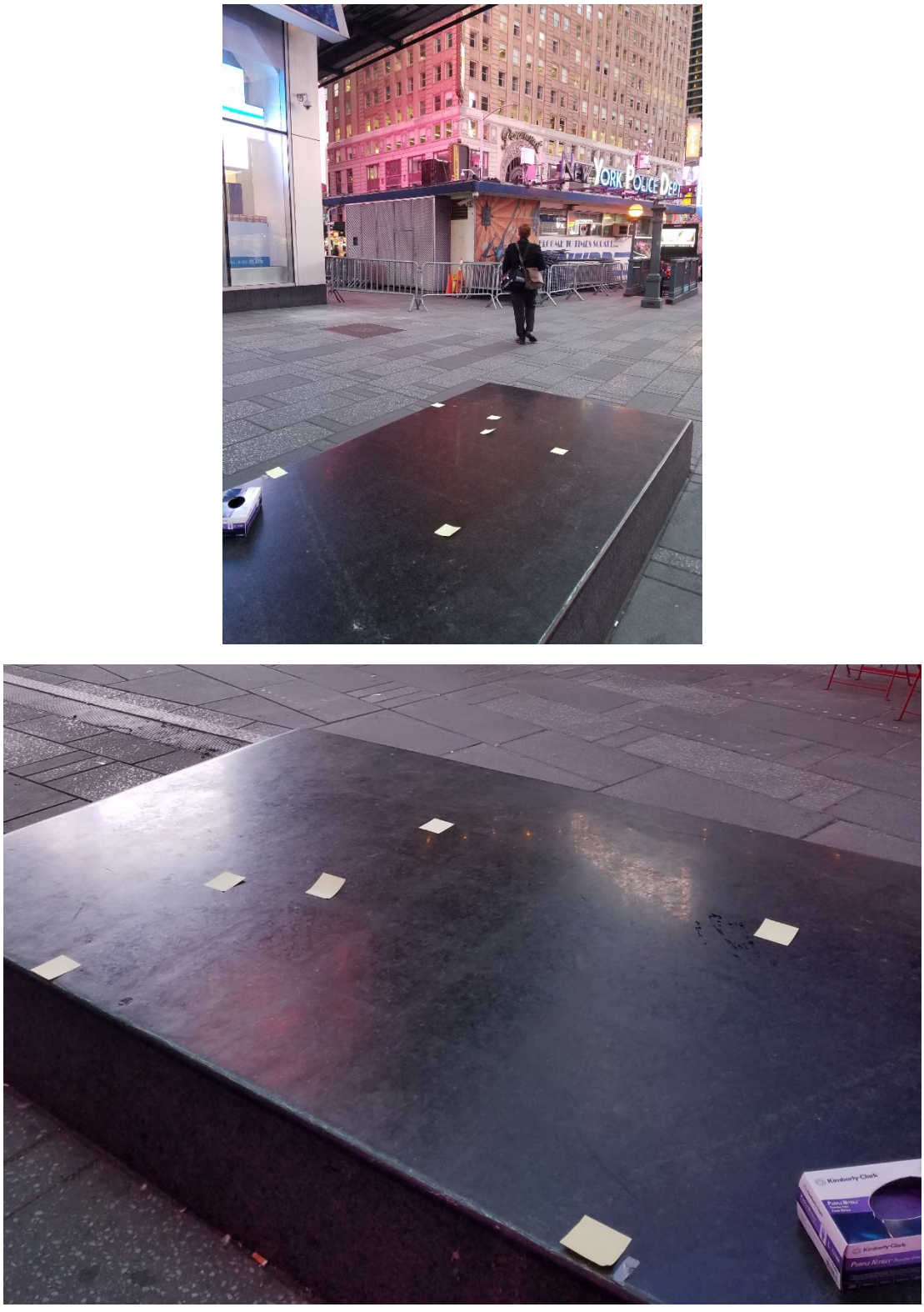


Figure A-10. Granite Bench Located Near the Times Square Police Station – Sampled with Sponge-Sticks

Figure A-11. Metro Card Machines Located Near the 42nd and Park Avenue Subway Exit – Sampled with Sponge-Sticks

Figure A-12. Overhead Signs Located at Various Locations Near the Grand Central Exit to 42nd Street and Madison Avenue – Sampled with Sponge-Sticks

Figure A-13. Crosswalk Painted Pavement (Asphalt) Located on the Intersection of Broadway and 46th Street – Sampled with Sponge-Sticks

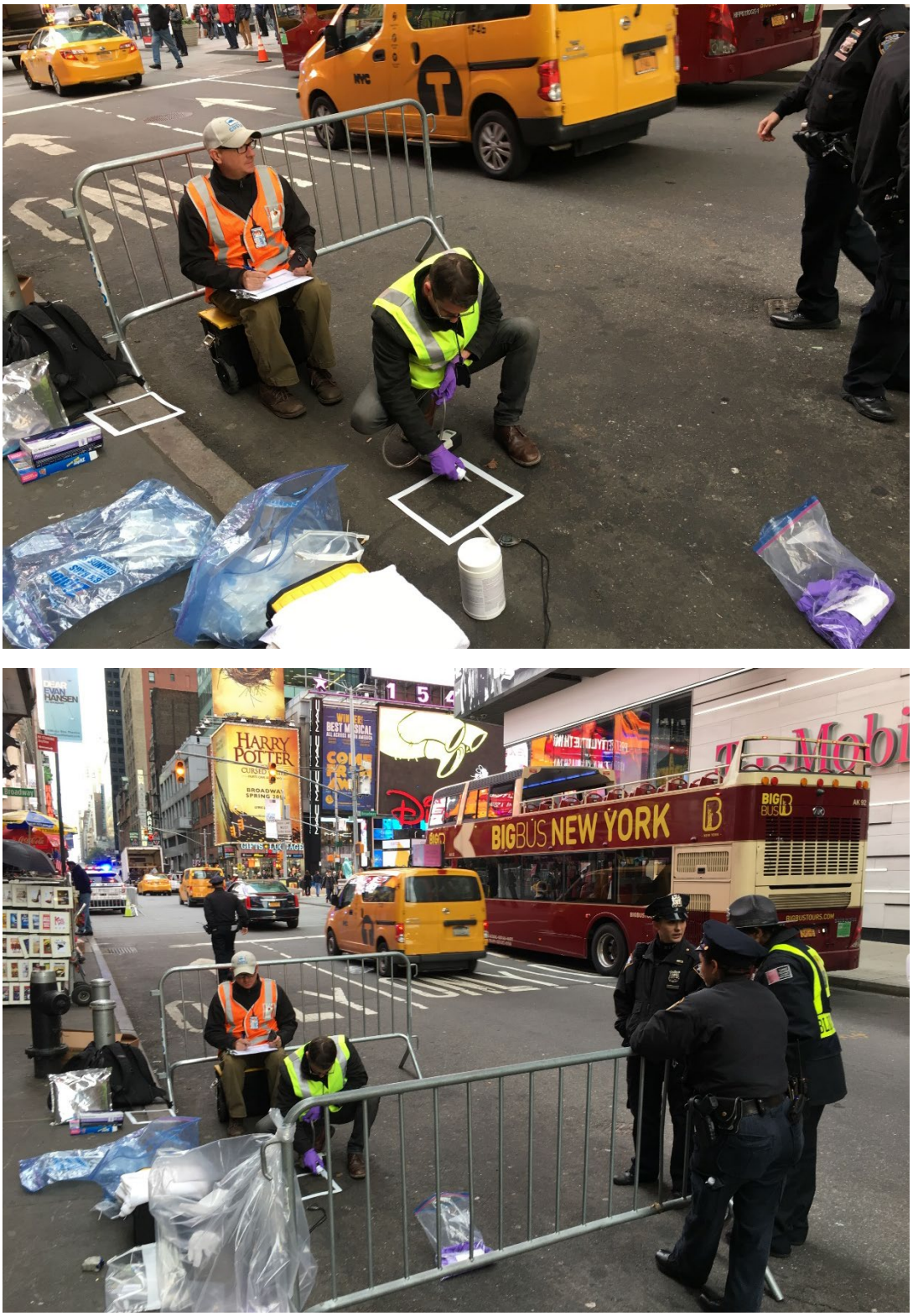


Figure A-14. Pavement (Asphalt) Located at the Intersection of Broadway and 46th Street – Sampled with Vacuum Filter Cassettes

Figure A-15. Sidewalk Concrete Located Next to the Green Wall by the Intersection of Broadway and 45th Street – Sampled with Vacuum Filter Cassettes and Sponge-Sticks

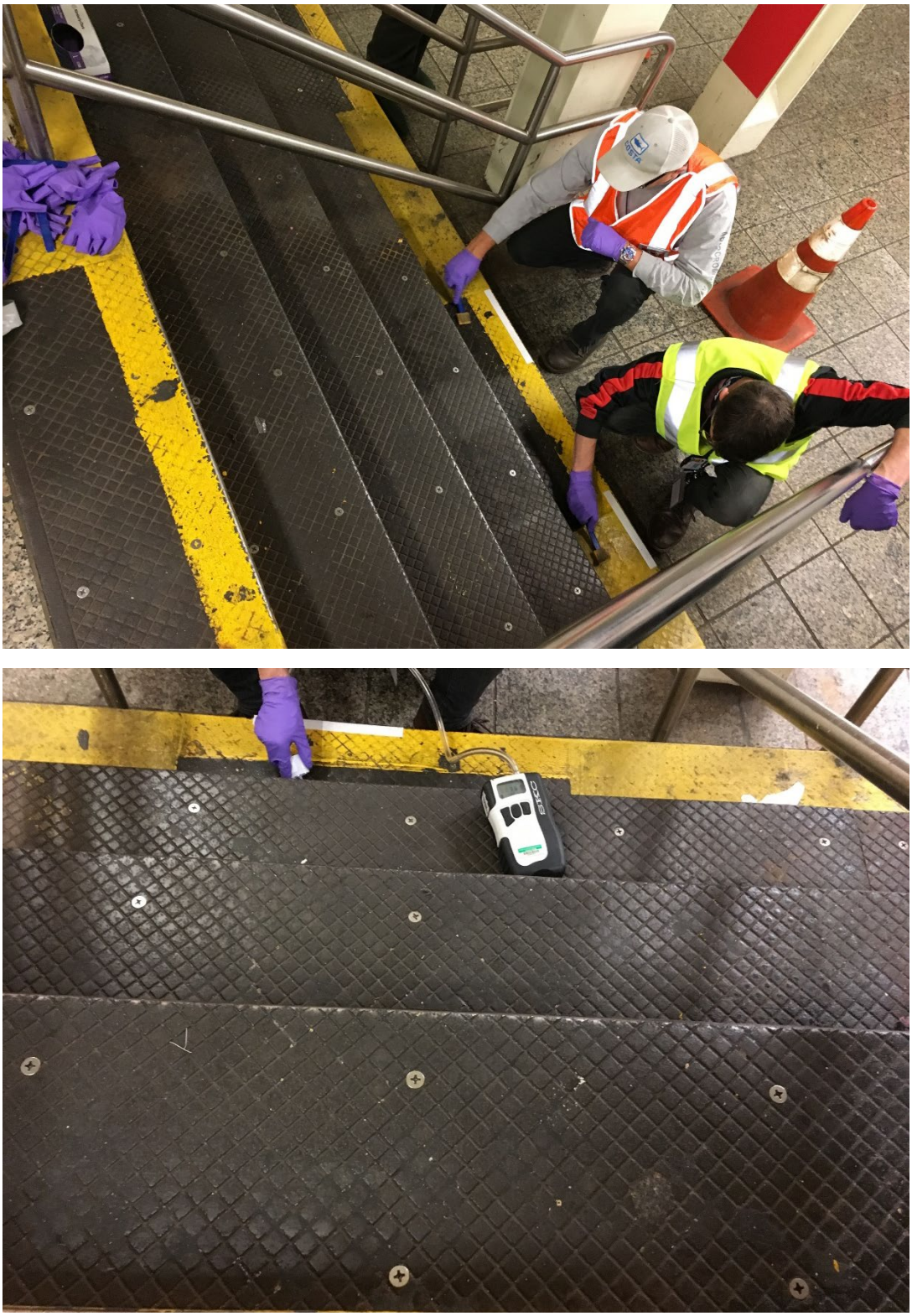


Figure A-16. Steps (Metal Grid) Located Near the PSU by the Record Mart – Sampled with Vacuum Filter Cassettes and Sponge-Sticks

Figure A-17. Street Grate Located by the Intersection of Broadway and 46th Street – Sampled with Sponge-Sticks

Figure A-18. Subway Car HVAC Filters Taken from the Front and Back HVAC Returns of Cars – Sampled with Vacuum Filter Cassettes (Offsite)

Figure A-19. Subway Car Filter Grille Located on the Front and Back HVAC Returns of Car 1951 – Sampled with Sponge-Sticks

Figure A-20. Telephone Booth (Interior) – Sampled with Sponge-Sticks

Figure A-21. Wall Tiles (Ceramic) Located Just Below the Times Square Police Station, One Level Down – Sampled with Sponge-Sticks

APPENDIX B: FORMULATIONS OF RECIPES USED IN BIOLOGICAL TEST METHODS

Spore Production

Table 1. Components of Modified G Sporulation Medium

Ingredient	Amount/L
Yeast Extract	2.0 g
$(NH_4)_2SO_4$	2.0 g
$CaCl_2 \bullet 2H_2O$	0.03 g
$CuSO_4 \bullet 5H_2O$	0.005 g
$FeSO_4 \bullet 7H_2O$	0.0005 g
$MgSO_4 \bullet 7H_2O$	0.2 g
$MnSO_4 \bullet H_2O$*	0.06 g
$ZnSO_4 \bullet 7H_2O$	0.005 g
K_2HPO_4	0.5 g
dH_2O	1000 mL

*$MnSO_4 \bullet H_2O$ substituted for $MnSO_4 \bullet 4H_2O$. If $MnSO_4 \bullet 4H_2O$ is used, add 0.05 g.

Table 2. Components of Leighton-Doi Sporulation Medium

Component	Amount/L
KCl	1.88 g
$CaCl_2$	0.29 g
$FeSO_4$ x 7 H_2O	0.003 g
$MnSO_4$ x H_2O	0.0017 g
$MgSO_4$ x 7 H_2O	0.025 g
Dextrose	0.9 g
Nutrient Broth	16.0 g

Table 3. Duplex Assay Conditions

Component (Duplex Assay)	**Volume for one reaction (µL)**
2x FAST PCR Mix	12.5
PCR-grade water	1.5
pXO1 For Primer (25 µM)	1
pXO1 Rev Primer (25 µM)	1
pXO1 Probe (2 µM)	1
chromosome For Primer (25 µM)	1
chromosome Rev Primer (25 µM)	1
chromosome Probe (2 µM)	1
Template	5
Total volume	**25**

APPENDIX C: WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES–SPG STICKS

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – SPG STICKS

I. PURPOSE/SCOPE

To spike Sponge-Sticks for spore recovery testing.

II. Analyst/Reviewers

Role	Name	Initials	Date
Analyst			
Analyst			
Reviewer			

III. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
Bacillus anthracis 34F2 spores	Inhouse	34F2101716	TBD	2-8 °C	
Sterile DI water	Teknova	W335019D1701	4/19/20	R.T.	
Blood Agar	BBL			2-8 °C	
1.5 or 2 mL tubes	Eppendorf	F170109Q G175420P	N/A	R.T.	
Sterile forceps	N/A	N/A	N/A	R.T.	

Equipment

Item	Manufacturer	Serial Number	Thermometer /Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company	57544	N/A	8/2019	
Micropipette Type:L1000	Rainin		N/A		
Micropipette Type:L200	Rainin		N/A		
Micropipette Type:L10 or L20	Rainin		N/A		
Refrigerator	Fisher	C3274822	115	3/2019	

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette filter tips
- Biohazard bags
- Bench coat
- Filters

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – SPG STICKS

IV. PROCEDURE

A. Decontaminate the BSC with DNA Erase, bleach and isopropanol prior to use.
   1. Decontaminated by _____ Date_____

B. Name filters
   1. Label the Stomacher bag with filter ID for each filter.
      i. AAA-BBB-CCC-DDD
         1. AAA = Sample #
         2. BBB = Sample Type
         3. CCC = Location
         4. DDD = Spore Spike Level
      ii. Electronically populate below table with sample names to be prepared on each day from the Sample Log.

Sample #	Sample Type	Location	Filter Vial Type	Spore Spike level	Filter ID	Date Spiked /initials
1	SPG	Field Blank	PVDF	0	1-SPG-FLDBLK-A-S15-0	
2	SPG	Street Grate	PVDF	0	2-SPG-STGRAT-A-S15-0	
3	SPG	Field Blank	PVDF	30	3-SPG-FLDBLK-A-S16-30	
4	SPG	Street Grate	PVDF	30	4-SPG-STGRAT-A-S16-30	
5	SPG	Field Blank	PVDF	300	5-SPG-FLDBLK-A-S17-300	
6	SPG	Street Grate	PVDF	300	6-SPG-STGRAT-A-S17-300	
7	SPG	Field Blank	PVDF	3000	7-SPG-FLDBLK-A-S18-3000	
8	SPG	Street Grate	PVDF	3000	8-SPG-STGRAT-A-S18-3000	
9	SPG	Granite Bench	PVDF	0	9-SPG-GRNBEN-A-S15-0	
10	SPG	Wall Tile	PVDF	0	10-SPG-WLTILE-B-S19-0	
11	SPG	Granite Bench	PVDF	30	11-SPG-GRNBEN-A-S16-30	
12	SPG	Lab Blank	PVDF	30	12-SPG-LABBLANK-30	
13	SPG	Granite Bench	PVDF	300	13-SPG-GRNBEN-A-S17-300	
14	SPG	Lab Blank	PVDF	300	14-SPG-LABBLANK-300	
15	SPG	Granite Bench	PVDF	3000	15-SPG-GRNBEN-A-S18-3000	
16	SPG	Lab Blank	PVDF	3000	16-SPG-LABBLANK-3000	

C. Spike Swatches
   1. Prepare dosing stocks
      i. Fill in information from stock tube.

Organism	Lot	Prep date	Concentration	Date of enumeration	Entered/verified by:
B. anthracis Sterne	34F2101716		1.8 X 10^8 cfu/mL	September 26, 2018	

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – SPG STICKS

ii. Target stock concentration(s).

Stock #	Organism	Lot	Prep date	Concentration	Total spores per 100 µL	Entered/verified by:
1	*B. anthracis* Sterne	34F2101716		3.0×10^4 cfu/mL	3,000	
2	*B. anthracis* Sterne	34F2101716		3.0×10^3 cfu/mL	300	
3	*B. anthracis* Sterne	34F2101716		3.0×10^2 cfu/mL	30	

iii. Prepare dilutions of stock in sterile DI water. Vortex stock on high for 30 seconds prior to preparing dilutions.
Show calculations:

$(1.8 \times 10^8$ cfu/mL)*(X)=$(3.0 \times 10^7$ cfu/mL)(1mL) → 167µL of sample into 833µL H20

$(3.0 \times 10^7$ cfu/mL)*(X)=$(3.0 \times 10^6$ cfu/mL)(1mL) → 100µL of sample into 900µL H20

$(3.0 \times 10^6$ cfu/mL)*(X)=$(3.0 \times 10^5$ cfu/mL)(1mL) → 100µL of sample into 900µL H20

$(3.0 \times 10^5$ cfu/mL)*(X)=$(3.0 \times 10^4$ cfu/mL)(1mL) → 150µL of sample into 1350µL H20

$(3.0 \times 10^4$ cfu/mL)*(X)=$(3.0 \times 10^3$ cfu/mL)(1mL) → 150µL of sample into 1350µL H20

$(3.0 \times 10^3$ cfu/mL)*(X)=$(3.0 \times 10^2$ cfu/mL)(1mL) → 150µL of sample into 1350µL H20

Dilutions Prepared By: _____ Date/Initials: _____

2. Spike Sponge Sticks
   i. Position sponge in specimen cup so that the dirty side is facing up. Change forceps between samples.
   ii. Prior to dosing filters, immediately vortex the stock for 30 seconds.
   iii. Per sponge, transfer a 120 µL aliquot of the appropriate Stock tube (Low, Med., or High) into a 1.5 ml tube.
   iv. Place ten 5 µL droplets onto each side of the sponge stick (twenty 5 µL droplets total), being as careful as possible to not have spiked surfaces contact the specimen cup wall. Position sponge stick as shown in Figure 1. The same pipet tip can be used to place all twenty droplets, dispose of the 120 µL aliquot once each swatch has been dosed.
   v. Seal the specimen cup and stock overnight @ 2 – 8 °C or process immediately to Spore recovery.
   Start time: _____ Date/Initials: _____
   End time: _____ Date/Initials: _____
3. Enumerate stock

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – SPG STICKS

i. Serially dilute the suspension in Sterile water (if necessary).
   1. Fill 2 mL dilution tubes for each sample with 900µL of Sterile water and label appropriately.
   2. Vortex the stock on high for 30 seconds.
   3. Transfer 100 µL of the stock into the first dilution tube containing 900µL of Sterile water. Recap the tube and vortex it on high for 30 seconds. This is the 10^{-1} suspension.

ii. Spread 100 µL aliquots of dilutions onto Blood Agar in triplicate.

iii. Incubate plates
   1. Invert the plates and incubate them at 37°C ± 2°C for 18 – 24 hours. *B. anthracis* produces flat or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a "ground glass" appearance.

   Incubation start Date/Time: _____ Initials: _____

   Incubation end Date/Time: _____ Initials: _____

iv. Plate counts
   1. Record counts in the below table.

Stock	Media Type	Volume/ (Dilution on Plate)	Plate Counts			Average Counts	CFU/mL
			Plate 1	Plate 2	Plate 3		
1 (3.0×10^4 cfu/mL)	Blood Agar	100 µL/ (10^{-1})					
2 (3.0×10^3 cfu/mL)	Blood Agar	100 µL/ (10^{-1})					
3 (3.0×10^2 cfu/mL)	Blood Agar	100 µL/ (10^{-1})					

Recorded By: _____ Date/Initials: _____

Position with folded side up or stick side up. Do not spike the sides of the sponge that could contact the specimen cup wall.
Place ten 5 µL evenly dispersed droplets on each side for a total of twenty 5 µL droplets.

Figure 1. Spiking diagram for sponge sticks.

APPENDIX D: WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES–VCF

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – VCF

I. PURPOSE/SCOPE

To spike 37 mm Vacuum Cassette Filters (VCF) for the spore recovery testing.

II. Analyst/Reviewers

Role	Name	Initials	Date
Analyst			
Analyst			
Reviewer			

III. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
Bacillus anthracis 34F2 spores	Inhouse	34F2101716	TBD	2-8 °C	
Sterile DI water	Teknova	W335017E1701	5/17/2020	R.T.	
Blood Agar	BBL	8313853	2/28/2019	2-8 °C	
15 mL tubes	Falcon	12118014	N/A	R.T.	
1.5 or 2 mL tubes	Eppendorf	H176955G	1/28/2023	R.T.	
100% Ethanol	Fisher	184834	11/9/2023	R.T.	

Equipment

Item	Manufacturer	Serial Number	Thermometer /Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company	57544	N/A	8/2019	
Micropipette Type:L1000	Rainin	C25845	N/A	4/23/2019	
Micropipette Type:L200	Rainin	C19039	N/A	2/28/2019	
Micropipette Type:L10 or L20	Rainin	C25835	N/A	6/12/2019	
Refrigerator	Fisher	C3274822	115	3/2019	

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette filter tips
- Biohazard bags
- Bench coat
- Filters

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – VCF

IV. PROCEDURE

A. Decontaminate the BSC with DNA Erase, bleach and isopropanol prior to use.
   1. Decontaminated by _____ Date_____
B. Name filters
   1. Label the Stomacher bag with filter ID for each filter.
      i. AAA-BBB-CCC-DDD
         1. AAA = Sample #
         2. BBB = Sample Type
         3. CCC = Location
         4. DDD = Spore Spike Level
      ii. Electronically populate below table with sample names to be prepared on each day from the Sample Log.

Sample #	Sample Type	Location	Filter Vial Type	Spore Spike level	Filter ID	Date Spiked /initials
1	VCF	Floor Conc.	PVDF	0	1-VCF-FLCON-B-S15-0	
2	VCF	Steps	PVDF	0	2-VCF-STEPS-B-S15-0	
3	VCF	Floor Conc.	PVDF	30	3-VCF-FLCON-B-S16-30	
4	VCF	Steps	PVDF	30	4-VCF-STEPS-B-S16-30	
5	VCF	Floor Conc.	PVDF	300	5-VCF-FLCON-B-S17-300	
6	VCF	Steps	PVDF	300	6-VCF-STEPS-B-S17-300	
7	VCF	Floor Conc.	PVDF	3,000	7-VCF-FLCON-B-S18-3,000	
8	VCF	Steps	PVDF	3,000	8-VCF-STEPS-B-S18-3,000	
9	VCF	Sidewalk Conc.	PVDF	0	9-VCF-SWCON-A-S15-0	
10	VCF	Pavement	PVDF	0	10-VCF-PAVEMT-A-S15-0	
11	VCF	Sidewalk Conc.	PVDF	30	11-VCF-SWCON-A-S16-30	
12	VCF	Pavement	PVDF	30	12-VCF-PAVEMT-A-S16-30	
13	VCF	Sidewalk Conc.	PVDF	300	13-VCF-SWCON-A-S17-300	
14	VCF	Pavement	PVDF	300	14-VCF-PAVEMT-A-S17-300	
15	VCF	Sidewalk Conc.	PVDF	3,000	15-VCF-SWCON-A-S18-3,000	
16	VCF	Pavement	PVDF	3,000	16-VCF-PAVEMT-A-S18-3,000	

C. Spike Swatches
   1. Prepare dosing stocks
      i. Fill in information from stock tube.

Organism	Lot	Prep date	Concentration	Date of enumeration	Entered/verified by:
B. anthracis Sterne	34F2101716		3.0 X 10^8 cfu/mL	January 21, 2019	

      ii. Target stock concentration(s).

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – VCF

Stock #	Organism	Lot	Prep date	Conc. in H_2O	Conc. in 50% Ethanol	Total spores per 100 µL Spike	Entered/verified by:
1	*B. anthracis* Sterne	34F2101716		6.0×10^4 cfu/mL	3.0×10^4 cfu/mL	3,000	
2	*B. anthracis* Sterne	34F2101716		6.0×10^3 cfu/mL	3.0×10^3 cfu/mL	300	
3	*B. anthracis* Sterne	34F2101716		6.0×10^2 cfu/mL	3.0×10^2 cfu/mL	30	

iii. Prepare dilutions of stock in sterile DI water. Vortex stock on high for 30 seconds prior to preparing dilutions.
Show calculations:

Dilution 1: $(3.0 \times 10^8$ cfu/mL)*(X)=$(3.0 \times 10^7$ cfu/mL)(1mL) → 100µL of sample into 900µL H_2O

Dilution 2: $(3.0 \times 10^7$ cfu/mL)*(X)=$(3.0 \times 10^6$ cfu/mL)(1mL) → 100µL of sample into 900µL H_2O

Dilution 3: $(3.0 \times 10^6$ cfu/mL)*(X)=$(3.0 \times 10^5$ cfu/mL)(1mL) → 100µL of sample into 900µL H_2O

Dilution 4: $(3.0 \times 10^5$ cfu/mL)*(X)=$(6.0 \times 10^4$ cfu/mL)(1.5mL) → 300µL of sample into 1200µL H_2O

Dilution 5: $(6.0 \times 10^4$ cfu/mL)*(X)=$(6.0 \times 10^3$ cfu/mL)(1.5mL) → 150µL of sample into 1350µL H_2O

Dilution 6: $(6.0 \times 10^3$ cfu/mL)*(X)=$(6.0 \times 10^2$ cfu/mL)(1.5mL) → 150µL of sample into 1350µL H_2O

Dilutions Prepared By: _____ Date/Initials: _____

iv. To prepare Stock #1 (3,000 Target Load Spike), add 500 µL of Dilution 4 and 500 µL 100% Ethanol to a 1.5 or 2 mL tube and vortex mix thoroughly.
v. To prepare Stock #2 (300 Target Load Spike), add 500 µL of Dilution 5 and 500 µL 100% Ethanol to a 1.5 or 2 mL tube and vortex mix thoroughly.
vi. To prepare Stock #3 (30 Target Load Spike), add 500 µL of Dilution 6 and 500 µL 100% Ethanol to a 1.5 or 2 mL tube and vortex mix thoroughly.

2. Spike VCF
   i. Wipe each cassette with 10% bleach solution or bleach wipes followed by a clean Kimwipe® and discard wipes into an autoclavable biohazard bag.
   ii. Prior to dosing filters, immediately vortex the stock for 30 seconds.
   iii. Per cassette, transfer a 120 µL aliquot of the appropriate Stock tube (1 High, 2 Med., or 3 Low) into a 1.5 mL tube.

iv. Remove the red plug and apply twenty 5 µL droplets onto each filter as shown in the below diagram. The same pipet tip can be used to place all twenty droplets, dispose of the remaining volume once each filter has been dosed.
v. Air dry in BSC overnight with the red plug removed.
Start time: _____ Date/Initials: _____
End time: _____ Date/Initials: _____
vi. Firmly replace the top section of each cassette.

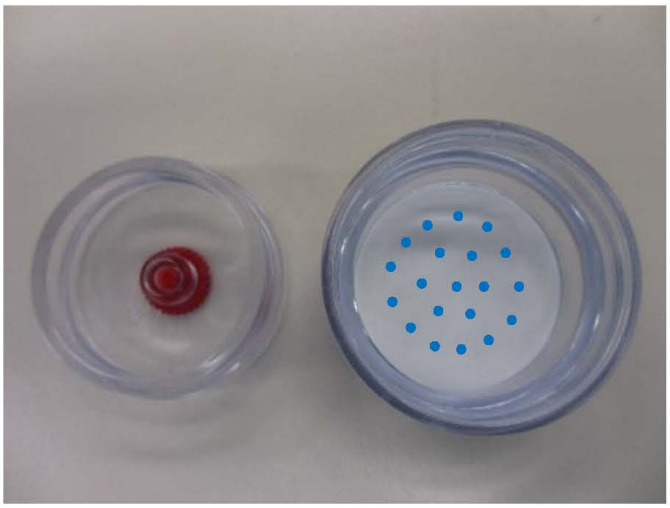
3. Enumerate stock
   i. Spread 100 µL aliquots of Dilution #5 and 6 onto Blood Agar in triplicate.
   ii. Incubate plates
      1. Invert the plates and incubate them at 37°C ± 2°C for 18 – 24 hours. *B. anthracis* produces flat or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a "ground glass" appearance.

      Incubation start Date/Time: _____ Initials: _____
      Incubation end Date/Time: _____ Initials: _____
   iii. Plate counts
      1. Record counts in the below table.

Dilution Tube	Media Type	Volume	Plate Counts			Average Counts	CFU In 50% Ethanol Stock
			Plate 1	Plate 2	Plate 3		
5 (6.0 X 10^3 cfu/mL)	Blood Agar	100 µL/					
6 (6.0 X 10^2 cfu/mL)	Blood Agar	100 µL/					

Recorded By: _____ Date/Initials: _____

WORK INSTRUCTION FOR SPIKING WITH *BACILLUS ANTHRACIS* STERNE SPORES – VCF



Place twenty 5 µL evenly dispersed droplets onto filter.

Figure 1. Spiking diagram for VCF.

APPENDIX E: WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY–SPG STICKS

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – SPG STICKS

I. PURPOSE/SCOPE

To recover *B. anthracis* spores from air filters following the **EPA/600/R-17/213** published by the EPA July 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
extraction buffer with Tween® 20	Inhouse			2-8 °C	
Stomacher Lab Blender Bags	Seward		N/A	RT	
Stomacher Bag Racks	Seward	BA6096	N/A	RT	
10X PBS	Teknova			2-8 °C	
1X PBS (pH 7.4)	Teknova			2-8 °C	
BHI broth	Inhouse			2-8 °C	
Conical tubes, 15 mL			N/A	R.T.	
Falcon Conical Tube, 50mL			N/A	R.T.	
Screw top flask, 250 mL	Corning		N/A	R.T.	
0.45 µm filter vials	Whatman		N/A	R.T.	
2mL screw cap tubes	VWR	70249-813C7-8102	N/A	R.T.	
Sterile disposable forceps					

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company	57553	N/A	8/2019	
		57544		8/2019	
Micropipette Type:L1000	Rainin		N/A		
Incubator Shaker	New Brunswick	590644988	C22712	1/25/19	
Refrigerator	Fisher	C3274822	115	3/2019	
Swinging Bucket Centrifuge	Beckman Coulter	X59221	N/A	N/A	
Stomacher	Seward	40142	N/A	N/A	

N/A = Not Applicable

Filters – Electronically update this table with samples names from the Sample Log

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – SPG STICKS

Sample #	Sample type	Sample Location	Filter Vial Type	Spore Spike level	Sample ID
1	SPG	Field Blank	PVDF	0	1-SPG-FLDBLK-A-S15-0
2	SPG	Street Grate	PVDF	0	2-SPG-STGRAT-A-S15-0
3	SPG	Field Blank	PVDF	30	3-SPG-FLDBLK-A-S16-30
4	SPG	Street Grate	PVDF	30	4-SPG-STGRAT-A-S16-30
5	SPG	Field Blank	PVDF	300	5-SPG-FLDBLK-A-S17-300
6	SPG	Street Grate	PVDF	300	6-SPG-STGRAT-A-S17-300
7	SPG	Field Blank	PVDF	3000	7-SPG-FLDBLK-A-S18-3000
8	SPG	Street Grate	PVDF	3000	8-SPG-STGRAT-A-S18-3000
9	SPG	Granite Bench	PVDF	0	9-SPG-GRNBEN-A-S15-0
10	SPG	Lab Blank	PVDF	0	10-SPG-LABBLANK-0
11	SPG	Granite Bench	PVDF	30	11-SPG-GRNBEN-A-S16-30
12	SPG	Lab Blank	PVDF	30	12-SPG-LABBLANK-30
13	SPG	Granite Bench	PVDF	300	13-SPG-GRNBEN-A-S17-300
14	SPG	Lab Blank	PVDF	300	14-SPG-LABBLANK-300
15	SPG	Granite Bench	PVDF	3000	15-SPG-GRNBEN-A-S18-3000
16	SPG	Lab Blank	PVDF	3000	16-SPG-LABBLANK-3000

Other Supplies and Equipment

- Forceps
- Biohazard bags
- Bleach
- 5 mL, 25 mL and 100 mL Serological Pipets
- Pipette aid
- Ziplock bags

III. PROCEDURE

A. RV-PCR Sample Processing: Spore Recovery for Sponge-Stick Samples

Note: Gloves should be used and changed between samples and as indicated below.

1. Prior to sample processing, prepare the following items:

- □ Fill sample tube rack with 50 mL screw cap conical tubes and label as appropriate, two 50 mL conical tubes are required per sample.
- □ In a BSC, attach the vacuum manifold to the vacuum trap, waste container (with 400 ml of bleach), and vacuum source. Attach the filter vials to the manifold, using outer rows first. Verify that all filter vials are completely pushed down. Place a red pull tab tapered plug in each filter vial.
- □ Document filter vial and sample tube labels.

Performed by: _____ Date:_____

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – SPG STICKS

- □ 1,500 mL of Extraction Buffer **with** Tween® 20 + Ethanol will be needed per set of 16 samples (90 mL per sample)
- □ 225 mL aliquot of High salt wash buffer (10x PBS) in a 250mL screw capped bottle per set of 16 samples (12.5 mL per sample).
- □ 225 mL aliquot of low salt wash buffer (1x PBS) in a 250mL screw capped bottle per set of 16 samples (12.5 mL per sample).

2. Add 90 mL cold (4°C) extraction buffer **with** Tween® 20 + Ethanol to each Stomacher bag.

3. Using sterile forceps, remove the remaining portion of the sponge stick handle and unfold the sponge.

4. Aseptically add a Sponge-Stick to a Stomacher bag. Open one bag at a time, close and seal bag prior to moving to the next sample. **Note: Save specimen cup for broth enrichment of sponge.**

5. Place an unsealed bag containing a sample into the Stomacher so the sponge rests evenly between the homogenizer paddles and stomach each sample for 1 minute at 260 rpm (Figure 1). Open the door of the Stomacher and remove the bag. Reseal bag.

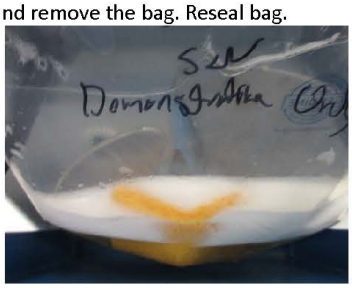


Figure 1. Sponge is opened and centered between paddle positions.

6. Stomach all sponges, removal of bag from Stomacher begins the settle time. Allow bags to sit for 10 minutes to allow elution suspension foam to settle.

7. Grab the sponge from the outside of the bag with hands. With the bag closed, move the sponge to the top of the bag while using hands to expel liquid from the sponge.

8. Open the bag, remove sponge and place into a labeled specimen cup using sterile forceps. Store sponge at 2 – 8 °C until enrichment in TSB (**See WI #7: TSB Enrichment for Culture Non-Detects**).

9. Follow steps described above for each sample, changing forceps between samples.

10. Gently mix the suspension in the Stomacher bag up and down three times with a sterile 50 mL pipet. Remove half of the suspension volume (~45-46 mL) and place it in a 50 mL screw cap centrifuge tube (Aliquot 1). Place the remaining suspension (~45-46 mL) into a second 50 mL tube (Aliquot 2). Adjust the suspension volumes so that volume is equal in both tubes.
11. Process the suspension for each sample, as described above.

Performed by: _____ Date:_____

12. Place 50 mL tubes into sealing centrifuge buckets and decontaminate centrifuge buckets before removing them from the BSC.

13. Centrifuge tubes at 3500 × g with the brake off, for 15 minutes in a swinging bucket rotor at 4°C.

14. Each sample has two pelleted aliquots (Aliquot 1 and Aliquot 2). Using a sterile 50 mL pipet, remove the supernatant from Aliquot 1 and discard it in an autoclavable leak-proof biohazard container. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube. Stop pipetting when meniscus reaches the 5 mL gradation level of on 50 mL Falcon tube, leaving ~2 – 3 mL in each tube. Next, using the same pipet, remove 20 mL of supernatant from Aliquot 2 and add it to Aliquot 1 pellet. Discard the remaining supernatant from Aliquot 2 into an autoclavable leak-proof biohazard container.

15. Vortex Aliquot 1 (containing ~22 mL of supernatant) for 30 seconds to resuspend the pellet, then transfer entire volume to Aliquot 2.

16. Vortex Aliquot 2 for 30 seconds to resuspend the pellet. This pooled suspension of ~25 mL will be used for culture and RV-PCR analytical methods. Record total volume for each sample in Table 1.

Table 1. Volume of sample recovered from Sponge Sticks.

Sample Number	Filter ID	Total volume recovered from Sponge-Stick	Recorded by:
1	1-SPG-FLDBLK-A-S15-0		
2	2-SPG-STGRAT-A-S15-0		
3	3-SPG-FLDBLK-A-S16-30		
4	4-SPG-STGRAT-A-S16-30		
5	5-SPG-FLDBLK-A-S17-300		
6	6-SPG-STGRAT-A-S17-300		
7	7-SPG-FLDBLK-A-S18-3000		
8	8-SPG-STGRAT-A-S18-3000		
9	9-SPG-GRNBEN-A-S15-0		
10	10-SPG-LABBLANK-0		
11	11-SPG-GRNBEN-A-S16-30		
12	12-SPG-LABBLANK-30		
13	13-SPG-GRNBEN-A-S17-300		
14	14-SPG-LABBLANK-300		
15	15-SPG-GRNBEN-A-S18-3000		
16	16-SPG-LABBLANK-3000		

17. Transfer 11 mL of the pooled extract and store on ice or in refrigerator until processed on same day using **WI #4: Culture of Recovered Spores**.

Performed by: _____ Date:_____

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – SPG STICKS

18. Place manifold and Whatman™ Autovial™ filter vials with red caps in BSC. Label all filter vials. Record filter vial labels.

19. Vortex each RV-PCR aliquot and allow 3 – 5 minutes of settle time to avoid loading large particulates into filter vial. Transfer 11 mL of the pooled suspension volume from each tube to the corresponding labeled filter vial by lifting red cap slightly. Change serological pipets and gloves between samples.

20. Complete filtration of liquid through filter vials. Turn off vacuum pump.

Note 1: At 15 minutes post-sample addition, if sample has not completely passed through the filter vial, a reduced volume of high salt wash buffer will be added (5 mL) to avoid prolonged filtering delays – it is desired to add a lower volume of each salt wash buffer (10X and 1X) than to omit one or both entirely.
Note 2: At 1 hour post-sample addition, if sample has not completely passed through the filter vial, the high salt and low wash steps will be omitted.

Sample #	Filter ID	Sample Addition		Volume of Wash Buffers		Recorded by:
		Start Time[1]	End Time[2]	10X	1X	
1	1-SPG-FLDBLK-A-S15-0					
2	2-SPG-STGRAT-A-S15-0					
3	3-SPG-FLDBLK-A-S16-30					
4	4-SPG-STGRAT-A-S16-30					
5	5-SPG-FLDBLK-A-S17-300					
6	6-SPG-STGRAT-A-S17-300					
7	7-SPG-FLDBLK-A-S18-3000					
8	8-SPG-STGRAT-A-S18-3000					
9	9-SPG-GRNBEN-A-S15-0					
10	10-SPG-LABBLANK-0					
11	11-SPG-GRNBEN-A-S16-30					
12	12-SPG-LABBLANK-30					
13	13-SPG-GRNBEN-A-S17-300					
14	14-SPG-LABBLANK-300					
15	15-SPG-GRNBEN-A-S18-3000					
16	16-SPG-LABBLANK-3000					

[1]Record the time of adding the final sample to filter vial.
[2]Record end time for samples that have clogging and meet the criteria in Notes 1 and 2 above.

21. Proceed to RV-PCR processing section (section B) below, with filter vial manifold.

B. **RV-PCR Sample Processing: Buffer Washes and Broth Culture**

1. Place into BSC: a ziplock bag with orange caps (one per filter vial), 10 mL serological pipets and cold (4°C) 10X PBS in 250 mL screw cap bottle.

Performed by: _____ Date:_____

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – SPG STICKS

2. Transfer 12.5 mL of cold (4°C) **High salt wash buffer (10x PBS)** to each filter-vial using a 10 mL serological pipet. Change pipet and gloves between each sample.

3. Complete filtration of liquid through the filter vials.

4. Place into the BSC: 10 mL serological pipets and cold (4°C) 1X low salt wash buffer in 250 mL screw cap bottle.

5. Transfer 12.5 mL cold (4°C) **low salt wash buffer (1x PBS)** to each filter-vial using a 10 mL serological pipet. Change pipet and gloves between each sample.

6. Complete filtration of liquid through filter vials. Turn off vacuum pump.

7. Using an Allen wrench, unscrew the top of the manifold and break the seal on manifold using a plate sealer to separate the top of the manifold.

8. Using a tray preloaded with caps, move the top of the manifold with the filters still in place and firmly press down, capping the bottoms of the filters. Repeat pressing down on each filter vial to ensure a good seal.

9. Place bleach soaked wipes onto the manifold to soak up the filtered waste and disinfect for 20 minutes.

10. Place into the BSC: 5 mL serological pipets, 1000 µL pipet, 1000 µL tips, cold (2-8°C) BHI broth aliquoted in 50 mL conical tubes, sharps container and orange caps.

11. Pipet 5 ml of **cold BHI broth** into each filter vial using a 5 mL serological pipet. Use a new pipet for each filter vial. Dispose of the red cap and place the orange cap firmly into the top of the filter. Change gloves between each sample.

12. Record the time of the BHI broth addition, this represents T_0. Bleach wipe the filter vial

    Time of BHI addition: _____

13. Place the rack of capped filter vials in a plastic bag, seal, double bag and bleach the bag.

14. Vortex the filter vials for 10 minutes on the platform vortexer, setting 7.

    Start time:_____ End Time:_____ Speed:_____

15. Place 2 mL screw cap tubes for T_0 aliquots onto ice in the BSC.

16. After vortexing, transfer filter vials to the BSC. Remove bag.

Performed by: _____ Date:_____

17. Uncap one filter vial at a time and open the corresponding 2 mL tube. Using a 1 mL pipette or serological pipet (if filter deteriorated), gently pipet up and down ~10 to mix. Transfer 1 mL from each vial to the corresponding pre-chilled (on ice) 2 mL screw cap tube for T_0. Cap the tube and place it back onto ice. Wipe the filter vial with a bleach soaked lab wipe. Change gloves between each sample

After transferring the T_0 aliquots for all samples, place the filter vial rack in a transfer container, seal, and bleach the container. Store the T_0 aliquot at -20 °C overnight.

T_0 -20 C storage start time: _____ End time: _____ Initial/Date:_____

18. Transfer the filter vial rack to the shaker incubator. Secure the rack. Incubate at 37ºC at 230 rpm, overnight (i.e., 16 hours from the addition of BHI broth to the filter vials). These samples are referred to as the T_i samples. Following incubation record turbidity observation and volume remaining in the table below.

Start time:_____ End Time:_____ Speed:_____ Temperature:_____

Sample Number	Filter ID	Turbid (Yes/No)	Volume remaining (mL)	Recorded by:
1	1-SPG-FLDBLK-A-S15-0			
2	2-SPG-STGRAT-A-S15-0			
3	3-SPG-FLDBLK-A-S16-30			
4	4-SPG-STGRAT-A-S16-30			
5	5-SPG-FLDBLK-A-S17-300			
6	6-SPG-STGRAT-A-S17-300			
7	7-SPG-FLDBLK-A-S18-3000			
8	8-SPG-STGRAT-A-S18-3000			
9	9-SPG-GRNBEN-A-S15-0			
10	10-SPG-LABBLANK-0			
11	11-SPG-GRNBEN-A-S16-30			
12	12-SPG-LABBLANK-30			
13	13-SPG-GRNBEN-A-S17-300			
14	14-SPG-LABBLANK-300			
15	15-SPG-GRNBEN-A-S18-3000			
16	16-SPG-LABBLANK-3000			

19. Proceed to WI #3: DNA Purification to process T_0 and T_i samples

IV. Technical Review

Performed by: _____ Date:_____

APPENDIX F: WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY–VCF

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – VCF

I. PURPOSE/SCOPE

To recover *B. anthracis* spores from air filters following the **EPA/600/R-17/213** published by the EPA July 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
Extraction Buffer with Tween® 20 + 30% Ethanol	In house			2-8 °C	
10X PBS	Teknova			2-8 °C	
1X PBS (pH 7.4)	Teknova			2-8 °C	
BHI broth	In house			2-8 °C	
Conical tubes, 15 mL	Falcon	12118014	N/A	R.T.	
Conical Tube, 50mL	N/A	N/A	N/A	R.T.	
Screw top flask, 250 mL			N/A	R.T.	
0.45 µm filter vials	Whatman		N/A	R.T.	
2mL screw cap tubes	VWR		N/A	R.T.	
2 oz. cups with lids (autoclaved)	Container & Packaging	N/A	N/A	R.T.	
Sterile Forceps	Unomedical			R.T.	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company	57553 57544	N/A	8/2019 8/2019	
Micropipette Type:L1000	Rainin	C25845 C20268	N/A	4/23/2019 6/12/2019	
Incubator Shaker	New Brunswick	590644988			
Refrigerator	Fisher	C3274822	115	3/2019	
Sonicator Bath	Bransonic	RNC010140514E	N/A	N/A	

N/A = Not Applicable

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – VCF

Filters – Electronically update this table with samples names from the Sample Log

Sample #	Sample Type	Location	Filter Vial Type	Spore Spike level	Filter ID
1	VCF	Floor Conc.	PVDF	0	1-VCF-FLCON-B-S15-0
2	VCF	Steps	PVDF	0	2-VCF-STEPS-B-S15-0
3	VCF	Floor Conc.	PVDF	30	3-VCF-FLCON-B-S16-30
4	VCF	Steps	PVDF	30	4-VCF-STEPS-B-S16-30
5	VCF	Floor Conc.	PVDF	300	5-VCF-FLCON-B-S17-300
6	VCF	Steps	PVDF	300	6-VCF-STEPS-B-S17-300
7	VCF	Floor Conc.	PVDF	3,000	7-VCF-FLCON-B-S18-3,000
8	VCF	Steps	PVDF	3,000	8-VCF-STEPS-B-S18-3,000
9	VCF	Sidewalk Conc.	PVDF	0	9-VCF-SWCON-A-S15-0
10	VCF	Pavement	PVDF	0	10-VCF-PAVEMT-A-S15-0
11	VCF	Sidewalk Conc.	PVDF	30	11-VCF-SWCON-A-S16-30
12	VCF	Pavement	PVDF	30	12-VCF-PAVEMT-A-S16-30
13	VCF	Sidewalk Conc.	PVDF	300	13-VCF-SWCON-A-S17-300
14	VCF	Pavement	PVDF	300	14-VCF-PAVEMT-A-S17-300
15	VCF	Sidewalk Conc.	PVDF	3,000	15-VCF-SWCON-A-S18-3,000
16	VCF	Pavement	PVDF	3,000	16-VCF-PAVEMT-A-S18-3,000

Other Supplies and Equipment

- Scissors
- Biohazard bags
- Bleach
- 5 mL, 25 mL and 100 mL Serological Pipets
- Pipette aid
- Ziplock bags
- Bench paper
- Stainless Steel SureSeal Cassette Opener, SKC cat. 225-13-5A

III. PROCEDURE

A. RV-PCR Sample Processing: Spore Recovery for Air Filter Samples

Note: Gloves should be used and changed between samples and as indicated below.

1. Prior to sample processing, prepare the following items:

- Fill sample tube rack with 15 mL screw cap conical tubes and label as appropriate, each containing 11 mL sterile Extraction Buffer **with** Tween® 20 + Ethanol.
- One labeled 2 oz. sterile cup with lid per sample, sterilized by autoclave (Gravity cycle, 121 °C for 15 minutes).

Performed by: _____ Date: _____

- In a BSC, attach the vacuum manifold to the vacuum trap, waste container (with 250 ml of bleach), and vacuum source. Attach the filter vials to the manifold, using outer rows first. Verify that all filter vials are completely pushed down. Place a red pull tab tapered plug in each filter vial.
- Document filter vial and sample tube labels.

2. For each 37-mm filter cassette, prepare one 15 mL conical tube containing 11 mL of sterile Extraction Buffer **with** Tween® 20 + Ethanol and label one 2 oz. sterile cup.

3. In the BSC remove the conical tube containing the nozzle and the cassette from the containment bags and wipe the outside of the conical tube with a disinfectant and place it into a rack. Aseptically add 5 mL of Extraction Buffer **with** Tween® 20 + Ethanol (from the 11 ml of a pre-measured aliquot of PBST + Ethanol) to the conical tube containing the nozzle and tubing and set aside.

4. Remove the band from around the cassette using sterile scissors. Wipe each cassette with 10% bleach solution or bleach wipes followed by a clean Kimwipe® and discard wipes into an autoclavable biohazard bag.

5. Change gloves. Remove the red plug from the inlet of the cassette; the plug on the back side should be kept in place. Using a pipette dispense 2 mL of Extraction Buffer **with** Tween® 20 + Ethanol from the tube now containing the 6 mL into the cassette and replace plug. Roll the cassette around to allow the liquid to touch all surfaces of the inside of the cassette. If there is a large quantity of particulate matter, more Extraction Buffer **with** Tween® 20 + Ethanol may be required. Particulate matter should be dampened enough to prevent aerosolization.

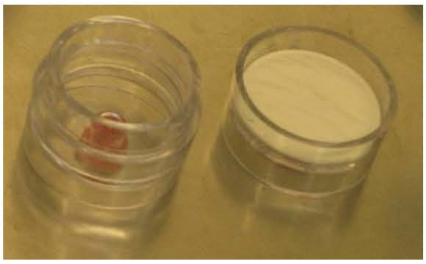
6. Using the cassette tool pry open the top section of the cassette, using care not to spill the Extraction Buffer **with** Tween® 20 + Ethanol inside the cassette and set aside, plug side down as shown in Figure 1. Set the bottom portion containing the filter aside carefully (filter side up), and using a pipette rinse the walls of the cassette with 2 mL of Extraction Buffer **with** Tween® 20 + Ethanol. Transfer the rinsate using the same pipette to the appropriately labelled 2 oz. sterile cup.

Figure 1. Vacuum Cassette with Top Section Removed

Performed by: _____ Date: _____

7. Using the cassette tool remove the middle section of the cassette (this piece is holding the filter in place) and place on top of top section as shown in Figure 2. Using sterile forceps aseptically remove the filter without picking up the support filter underneath. Place the filter in the 2 oz. cup with the rinsates.

Figure 2. Vacuum Cassette with Top and Middle Sections Removed



8. Use the remainder of the 6 mL Extraction Buffer **with** Tween® 20 + Ethanol to rinse walls of the middle and top sections (configuration shown in Figure 2, image on left) of the cassette and transfer rinsate to 2 oz. cup. Discard the cassette sections, support filter, plugs, and transfer pipette in an autoclavable biohazard bag.

9. Disinfect the outside of the 2 oz. cup with 10% bleach solution and place in tray. Decontaminate the BSC with 10% bleach solution and don a fresh pair of gloves in between samples. Repeat procedure described above for each 37-mm filter cassette.

10. Seal the conical tubes containing 5 mL Extraction Buffer **with** Tween® 20 + Ethanol, tubing and nozzle with Parafilm®. Place the rack of conical tubes into the sonicating bath to a level that allows at least 1 inch (~2.5 cm) of tube to be above the water line. Place a weight on top of the tubes to prevent them from floating or tipping over. Sonicate for 1 minute and remove tubes from the sonicating bath. Dry and disinfect each tube with a 10% bleach solution.

11. Vortex the conical tubes 2 minutes using platform vortex at Setting 10 (high setting), then transfer the 5 mL Extraction Buffer **with** Tween® 20 + Ethanol to the appropriate 2 oz. cup. To Transfer volume, use 1 mL micropipette to remove volume collected in the tubing nozzle, then use pipette tip to remove nozzle from the 15 mL conical tube. Before disposing of nozzle, depress pipette piston to expel any remaining extract volume from the nozzle into the 15 mL conical tube (See Figure 3).

Figure 3. Nozzle Removal Using 1 mL Pipette

Performed by: _____ Date: _____

12. Seal all of the 2 oz. cups with Parafilm®. Place the rack of 2 oz. cups in the sonicating bath and cover with a weight on top of the cups to prevent them from floating or tipping over. There should be 1 inch (~2.5 cm) between the level of the water and the cup lids. Sonicate for 3 minutes without heat. Remove rack from the bath and dry each cup with a Kimwipe® and place in the BSC. Place cups in a sealable plastic lidded box.

13. Using a 10 mL serological pipet, transfer as much suspension as possible from each 2 oz. cup to a 15 mL conical tube. Record total volume for each sample in Table 1. **Note: Save 2 oz. cups containing filter.** Store at 2 – 8 °C until enrichment in TSB on same day (**See WI #7: VCF TSB Enrichment for Culture**).

Table 1. Volume of sample recovered from VCF.

Sample Number	Filter ID	Total volume recovered from VCF	Volume available per analytical method (Total Volume ÷ 2)	Recorded by:
1	1-VCF-FLCON-B-S15-0			
2	2-VCF-STEPS-B-S15-0			
3	3-VCF-FLCON-B-S16-30			
4	4-VCF-STEPS-B-S16-30			
5	5-VCF-FLCON-B-S17-300			
6	6-VCF-STEPS-B-S17-300			
7	7-VCF-FLCON-B-S18-3,000			
8	8-VCF-STEPS-B-S18-3,000			
9	9-VCF-SWCON-A-S15-0			
10	10-VCF-PAVEMT-A-S15-0			
11	11-VCF-SWCON-A-S16-30			
12	12-VCF-PAVEMT-A-S16-30			
13	13-VCF-SWCON-A-S17-300			
14	14-VCF-PAVEMT-A-S17-300			
15	15-VCF-SWCON-A-S18-3,000			
16	16-VCF-PAVEMT-A-S18-3,000			

14. Vortex each sample, then allow 3 – 5 minutes settle time to avoid transferring large particulates into filter vial and cause clogging. Transfer half (~5 mL) volume of each sample to corresponding labeled filter vial. Change serological pipets and gloves between each sample.

15. Store the remaining half (~5 mL) of the pooled extract for microbiological analysis (**WI #4: Culture of Recovered Spores**). Store aliquot on ice or in refrigerator until processed on same day. Change serological pipets and gloves between each sample.

Performed by: _____ Date: _____

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – VCF

16. Complete filtration of liquid through filter vials. Turn off vacuum pump.

Note 1: At 15 minutes post-sample addition, if sample has not completely passed through the filter vial, a reduced volume of high salt wash buffer will be added (5 mL) to avoid prolonged filtering delays – it is desired to add a lower volume of each salt wash buffer (10X and 1X) than to omit one or both entirely.
Note 2: At 1 hour post-sample addition, if sample has not completely passed through the filter vial, the high salt and low wash steps will be omitted.

Sample #	Filter ID	Sample Addition		Volume of Wash Buffers		Recorded by:
		Start Time[1]	End Time[2]	10X	1X	
1	1-VCF-FLCON-B-S15-0					
2	2-VCF-STEPS-B-S15-0					
3	3-VCF-FLCON-B-S16-30					
4	4-VCF-STEPS-B-S16-30					
5	5-VCF-FLCON-B-S17-300					
6	6-VCF-STEPS-B-S17-300					
7	7-VCF-FLCON-B-S18-3,000					
8	8-VCF-STEPS-B-S18-3,000					
9	9-VCF-SWCON-A-S15-0					
10	10-VCF-PAVEMT-A-S15-0					
11	11-VCF-SWCON-A-S16-30					
12	12-VCF-PAVEMT-A-S16-30					
13	13-VCF-SWCON-A-S17-300					
14	14-VCF-PAVEMT-A-S17-300					
15	15-VCF-SWCON-A-S18-3,000					
16	16-VCF-PAVEMT-A-S18-3,000					

[1]Record the time of adding the final sample to filter vial.
[2]Record end time for samples that have clogging and meet the criteria in Notes 1 and 2 above.

B. **RV-PCR Sample Processing: Buffer Washes and Broth Culture**

1. Place into BSC: a ziplock bag with orange caps (one per filter vial), 10 mL serological pipets and cold (4°C) 10X PBS in 250 mL screw cap bottle.

2. Transfer 12.5 mL of cold (4°C) **High salt wash buffer (10x PBS)** to each filter-vial using a 10 mL serological pipet. Change pipet and gloves between each sample.

3. Complete filtration of liquid through the filter vials.

4. Place into the BSC: 10 mL serological pipets and cold (4°C) 1X low salt wash buffer in 250 mL screw cap bottle.

Performed by: _____ Date: _____

WORK INSTRUCTION FOR *BACILLUS ANTHRACIS* STERNE SPORE RECOVERY – VCF

5. Transfer 12.5 mL cold (4°C) **low salt wash buffer (1x PBS)** to each filter-vial using a 10 mL serological pipet. Change pipet and gloves between each sample.

6. Complete filtration of liquid through filter vials. Turn off vacuum pump.

7. Using an Allen wrench, unscrew the top of the manifold and break the seal on manifold using a plate sealer to separate the top of the manifold.

8. Using a tray preloaded with caps, move the top of the manifold with the filters still in place and firmly press down, capping the bottoms of the filters. Repeat pressing down on each filter vial to ensure a good seal.

9. Place bleach soaked wipes onto the manifold to soak up the filtered waste and disinfect for 20 minutes.

10. Place into the BSC: 5 mL serological pipets, 1000 µL pipet, 1000 µL tips, cold (2-8°C) BHI broth aliquoted in 50 mL conical tubes, sharps container and orange caps.

11. Pipet 5 ml of **cold BHI broth** into each filter vial using a 5 mL serological pipet. Use a new pipet for each filter vial. Dispose of the red cap and place the orange cap firmly into the top of the filter. Change gloves between each sample.

12. Record the time of the BHI broth addition, this represents T_0. Bleach wipe the filter vial

    Time of BHI addition: _____

13. Place the rack of capped filter vials in a plastic bag, seal, double bag and bleach the bag.

14. Vortex the filter vials for 10 minutes on the platform vortexer, setting 7.

    Start time:_____ End Time:_____ Speed:_____

15. Place 2 mL screw cap tubes for T_0 aliquots onto ice in the BSC.

16. After vortexing, transfer filter vials to the BSC. Remove bag.

17. Uncap one filter vial at a time and open the corresponding 2 mL tube. Using a 1 mL pipette or serological pipet (if filter deteriorated), gently pipet up and down ~10 to mix. Transfer 1 mL from each vial to the corresponding pre-chilled (on ice) 2 mL screw cap tube for T_0. Cap the tube and place it back onto ice. Wipe the filter vial with a bleach soaked lab wipe. Change gloves between each sample

    After transferring the T_0 aliquots for all samples, place the filter vial rack in a transfer container, seal, and bleach the container. Store the T_0 aliquot at -20 °C overnight.

    T_0 -20 C storage start time: _____ End time: _____ Initial/Date:_____
    Performed by: _____ Date: _____

18. Transfer the filter vial rack to the shaker incubator. Secure the rack. Incubate at 37ºC at 230 rpm, overnight (i.e., 16 hours from the addition of BHI broth to the filter vials). These samples are referred to as the T_{final} samples. Following incubation record turbidity observation and volume remaining in the table below.

Start time:_____ End Time:_____ Speed:_____ Temperature:_____

Sample Number	Filter ID	Turbid (Yes/No)	Volume remaining (mL)	Recorded by:
1	1-VCF-FLCON-B-S15-0			
2	2-VCF-STEPS-B-S15-0			
3	3-VCF-FLCON-B-S16-30			
4	4-VCF-STEPS-B-S16-30			
5	5-VCF-FLCON-B-S17-300			
6	6-VCF-STEPS-B-S17-300			
7	7-VCF-FLCON-B-S18-3,000			
8	8-VCF-STEPS-B-S18-3,000			
9	9-VCF-SWCON-A-S15-0			
10	10-VCF-PAVEMT-A-S15-0			
11	11-VCF-SWCON-A-S16-30			
12	12-VCF-PAVEMT-A-S16-30			
13	13-VCF-SWCON-A-S17-300			
14	14-VCF-PAVEMT-A-S17-300			
15	15-VCF-SWCON-A-S18-3,000			
16	16-VCF-PAVEMT-A-S18-3,000			

19. Proceed to WI #3: DNA Purification to process T_0 and T_i samples

Performed by: _____ Date: _____

IV. Technical Review

Performed by: _____ Date:_____

APPENDIX G: WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM SPG STICKS

WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM AIR FILTERS – SPG STICKS

I. PURPOSE/SCOPE

Culture of *B. anthracis* spores recovered from air filters following the **EPA/600/R-17/213** published by the EPA July 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
PBS with Tween (0.05%)	Teknova			2-8 °C	
Microfunnel filters	PALL			R.T.	
Blood Agar	BBL			2-8 °C	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company		N/A		
Stationary Incubator	Precision	9509-003	N/A	N/A	
Vacuum manifold	Gelman Sciences	N/A	N/A	N/A	

N/A = Not Applicable

Other Supplies and Equipment

- Forceps
- Bleach
- 5 mL, 10 mL, and 25mL Serological Pipettes
- Pipette aid

WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM AIR FILTERS – SPG STICKS

Filters – Electronically update this table with samples names from the Sample Log

Sample #	Sample type	Sample Location	Filter Vial Type	Spore Spike level	Sample ID
1	SPG	Field Blank	PVDF	0	1-SPG-FLDBLK-A-S15-0
2	SPG	Street Grate	PVDF	0	2-SPG-STGRAT-A-S15-0
3	SPG	Field Blank	PVDF	30	3-SPG-FLDBLK-A-S16-30
4	SPG	Street Grate	PVDF	30	4-SPG-STGRAT-A-S16-30
5	SPG	Field Blank	PVDF	300	5-SPG-FLDBLK-A-S17-300
6	SPG	Street Grate	PVDF	300	6-SPG-STGRAT-A-S17-300
7	SPG	Field Blank	PVDF	3000	7-SPG-FLDBLK-A-S18-3000
8	SPG	Street Grate	PVDF	3000	8-SPG-STGRAT-A-S18-3000
9	SPG	Granite Bench	PVDF	0	9-SPG-GRNBEN-A-S15-0
10	SPG	Lab Blank	PVDF	0	10-SPG-LABBLANK-0
11	SPG	Granite Bench	PVDF	30	11-SPG-GRNBEN-A-S16-30
12	SPG	Lab Blank	PVDF	30	12-SPG-LABBLANK-30
13	SPG	Granite Bench	PVDF	300	13-SPG-GRNBEN-A-S17-300
14	SPG	Lab Blank	PVDF	300	14-SPG-LABBLANK-300
15	SPG	Granite Bench	PVDF	3000	15-SPG-GRNBEN-A-S18-3000
16	SPG	Lab Blank	PVDF	3000	16-SPG-LABBLANK-3000

III. PROCEDURE

Note: The following procedure is to be carried out with the 10 mL pooled extract taken from step 17 (refer to WI #2 for Bacillus anthracis spore recovery). Process 2-3 PBST only negative control filter funnels alongside samples.

A. Culture Method

1. Label two filter funnels per sample, one with 2 mL and one with 8 mL.
2. Place the filter funnels onto the vacuum manifold in a Class II BSC.
3. Add 5 mL of PBS with 0.05% Tween (PBST) to each filter funnel. Apply vacuum.
4. With the vacuum valve closed and the vacuum pressure released, place 10 mL of PBST into each filter cup.
5. Vortex each sample, then allow 3 – 5 minutes of settle time to avoid loading large particulates into filter funnel. For each sample, add 2 mL of pooled extract to one filter funnel and 8 mL of pooled extract to one filter funnel. Apply vacuum. **Save remaining ~1 mL of culture aliquot and store at 2 – 8 °C until processing using WI #7: TSB Enrichment for Culture Non-Detects.**

Performed by: _____ Date: _____

WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM AIR FILTERS – SPG STICKS

6. Close the vacuum valve and release the vacuum pressure. Rinse the walls of each filter funnel using 10 mL of PBST. Apply vacuum.

7. With the vacuum valve closed and the vacuum pressure released, remove the membrane from the filter funnel and place onto Blood Agar. Dispose of filter bases and then change glove.

8. Incubate plates inverted overnight at 37°C ± 2°C.
Incubation start Date/Time: _____ Initials: _____
Incubation end Date/Time: _____ Initials: _____

9. Enter results into the below table.

Filter ID	*B. anthracis* colonies		Total colonies (all morphologies)	
	CFU/ 2 mL	CFU/ 8 mL	CFU/ 2 mL	CFU/ 8 mL
PBST Negative #1				
PBST Negative #2				
1-SPG-FLDBLK-A-S15-0				
2-SPG-STGRAT-A-S15-0				
3-SPG-FLDBLK-A-S16-30				
4-SPG-STGRAT-A-S16-30				
5-SPG-FLDBLK-A-S17-300				
6-SPG-STGRAT-A-S17-300				
7-SPG-FLDBLK-A-S18-3000				
8-SPG-STGRAT-A-S18-3000				
9-SPG-GRNBEN-A-S15-0				
10-SPG-LABBLANK-0				
11-SPG-GRNBEN-A-S16-30				
12-SPG-LABBLANK-30				
13-SPG-GRNBEN-A-S17-300				
14-SPG-LABBLANK-300				
15-SPG-GRNBEN-A-S18-3000				
16-SPG-LABBLANK-3000				

Counts performed/recorded by: _____ Date: _____

Performed by: _____ Date: _____

IV. Technical Review

Reviewed by: _____ Date: _____

APPENDIX H: WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM VCF

WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM AIR FILTERS – VCF

I. PURPOSE/SCOPE

Culture of *B. anthracis* spores recovered from air filters following the **EPA/600/R-17/213** published by the EPA July 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
PBS with Tween (0.05%)	Teknova			2-8 °C	
Microfunnel filters	PALL			R.T.	
Blood Agar	BBL			2-8 °C	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company		N/A		
Stationary Incubator	Precision	9509-003	N/A	N/A	
Vacuum manifold	Gelman Sciences	N/A	N/A	N/A	

N/A = Not Applicable

Other Supplies and Equipment

- Forceps
- Bleach
- 5 mL, 10 mL, and 25mL Serological Pipettes
- Pipette aid

WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM AIR FILTERS – VCF

Filters – Electronically update this table with samples names from the Sample Log

Sample #	Sample type	Sample Location	Filter Vial Type	Spore Spike level	Sample ID
1	VCF	Floor Conc.	PVDF	0	1-VCF-FLCON-B-S15-0
2	VCF	Steps	PVDF	0	2-VCF-STEPS-B-S15-0
3	VCF	Floor Conc.	PVDF	30	3-VCF-FLCON-B-S16-30
4	VCF	Steps	PVDF	30	4-VCF-STEPS-B-S16-30
5	VCF	Floor Conc.	PVDF	300	5-VCF-FLCON-B-S17-300
6	VCF	Steps	PVDF	300	6-VCF-STEPS-B-S17-300
7	VCF	Floor Conc.	PVDF	3,000	7-VCF-FLCON-B-S18-3,000
8	VCF	Steps	PVDF	3,000	8-VCF-STEPS-B-S18-3,000
9	VCF	Sidewalk Conc.	PVDF	0	9-VCF-SWCON-A-S15-0
10	VCF	Pavement	PVDF	0	10-VCF-PAVEMT-A-S15-0
11	VCF	Sidewalk Conc.	PVDF	30	11-VCF-SWCON-A-S16-30
12	VCF	Pavement	PVDF	30	12-VCF-PAVEMT-A-S16-30
13	VCF	Sidewalk Conc.	PVDF	300	13-VCF-SWCON-A-S17-300
14	VCF	Pavement	PVDF	300	14-VCF-PAVEMT-A-S17-300
15	VCF	Sidewalk Conc.	PVDF	3,000	15-VCF-SWCON-A-S18-3,000
16	VCF	Pavement	PVDF	3,000	16-VCF-PAVEMT-A-S18-3,000

III. PROCEDURE

Note: The following procedure is to be carried out with the ~5 mL extract taken from step 15 (refer to WI #2 for Bacillus anthracis spore recovery). Process 2-3 PBST only negative control filter funnels alongside samples.

A. Culture Method

1. Label two filter funnels per sample, one with 1 mL and one with 4 mL.
2. Place the filter funnels onto the vacuum manifold in a Class II BSC.
3. Add 5 mL of PBS with 0.05% Tween (PBST) to each filter funnel. Apply vacuum.
4. With the vacuum valve closed and the vacuum pressure released, place 10 mL of PBST into each filter cup.
5. Vortex each sample, then allow 3 – 5 minutes of settle time to avoid loading large particulates into filter funnel. For each sample, add 1 mL of pooled extract to one filter funnel and ~4 mL (remaining volume) of pooled extract to one filter funnel. Apply vacuum. Enter the remaining volume added to the 4 mL aliquot in below table.

Performed by: _____ Date: _____

WORK INSTRUCTION FOR CULTURE OF *BACILLUS ANTHRACIS* SPORES RECOVERED FROM AIR FILTERS – VCF

6. Close the vacuum valve and release the vacuum pressure. Rinse the walls of each filter funnel using 10 mL of PBST. Apply vacuum.

7. With the vacuum valve closed and the vacuum pressure released, remove the membrane from the filter funnel and place onto Blood Agar. Dispose of filter bases and then change glove.

8. Incubate plates inverted overnight at 37°C ± 2°C.
Incubation start Date/Time: _____ Initials: _____
Incubation end Date/Time: _____ Initials: _____

9. Enter results into the below table.

Filter ID	*B. anthracis* colonies		Total colonies (all morphologies)		Remaining Volume (4 mL Aliquot)
	CFU/ 1 mL	CFU/ 4 mL	CFU/ 1 mL	CFU/ 4 mL	
PBST Negative #1					N/A
PBST Negative #2					N/A
1-VCF-FLCON-B-S15-0					
2-VCF-STEPS-B-S15-0					
3-VCF-FLCON-B-S16-30					
4-VCF-STEPS-B-S16-30					
5-VCF-FLCON-B-S17-300					
6-VCF-STEPS-B-S17-300					
7-VCF-FLCON-B-S18-3,000					
8-VCF-STEPS-B-S18-3,000					
9-VCF-SWCON-A-S15-0					
10-VCF-PAVEMT-A-S15-0					
11-VCF-SWCON-A-S16-30					
12-VCF-PAVEMT-A-S16-30					
13-VCF-SWCON-A-S17-300					
14-VCF-PAVEMT-A-S17-300					
15-VCF-SWCON-A-S18-3,000					
16-VCF-PAVEMT-A-S18-3,000					

Counts performed/recorded by: _____ Date: _____

Performed by: _____ Date: _____

IV. Technical Review

Reviewed by: _____ Date:_____

APPENDIX I: WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS ANTHRACIS* SPORES

WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS ANTHRACIS* SPORES - RWI

I. PURPOSE/SCOPE

Manual DNA extraction and purification *B. anthracis* spores from recovered from air filters following the ***BACILLUS Analytical Methods 004*** published by the EPA December 2012.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
Lysis Buffer	Promega			RT	
PMPs	Promega			RT	
Salt Wash solution	Promega			RT	
Alcohol Wash	Promega			RT	
70% Ethanol	Inhouse			RT	
Elution Buffer	Promega			RT	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer /Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company	57544	N/A	8/2019	
Micropipette Type:L200	Rainin		N/A		
Micropipette Type:L200	Rainin		N/A		
Micropipette Type:L1000	Rainin		N/A		
Micropipette Type:L1000	Rainin		N/A		
Ultra-low Freezer	Woods	X34664	10	4/1/18	
Refrigerator	Thermo Fisher	35840	115		
Centrifuge	Eppendorf	X58983	N/A	N/A	
Heat block	VWR	949039	N/A	N/A	
Thermometer			N/A		

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette tips
- Biohazard bags
- Bleach
- Prepare tubes

WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS ANTHRACIS* SPORES - RWI

III. PROCEDURE

A. Manual DNA Extraction and Purification

Prepare lysis buffer with anti-foam according to manufacturer's instructions in the Magnesil Blood Genomic, Max Yield System, Kit. Prepare the alcohol wash solution by adding ethanol and isopropyl alcohol according to manufacturer's instructions. Prepare 70% Ethanol by adding 6 mL sterile water to 14 mL EtOH. Transfer sufficient volume of buffer to sterile, 100 mL reservoir immediately before use. ***Pre-heat heat block to 80°C prior to Section 10.4.8.***

NOTE: Process samples from zero spike level to 3,000 spike level. Change gloves when moving from a spiked sample to a sample containing a lower spike level, or if contamination of gloves is suspected. Pre-aliquot reagents from the kit to prevent contamination of reagents between runs.

1. After the overnight (16 h) incubation, remove the filter vial manifold from the shaker incubator. Thaw T_0 aliquots if they were stored at -20°C.

2. Vortex filter vials for 10 minutes on platform vortexer with speed set to 7.
   Start: _____ End: _____ Speed: _____

3. Transfer the filter vial manifold to the BSC, remove and discard bags.

4. Set up 2 mL screw cap tubes for T_i aliquots in a tube. Do not use 1.5 mL tubes. Transfer T_i aliquot screw cap tubes to the BSC.

5. Transfer the filter vial rack to the BSC. Uncap one filter vial at a time and transfer 1 mL to corresponding 2 mL tube after gently pipetting up and down ~10 to mix. Change gloves in between each sample.

6. Centrifuge 2 mL screw cap tubes (both T_0 and T_i) at 14,000 rpm for 10 minutes (4°C).

   Start: _____ End: _____ Speed: _____

7. Remove 800 µL of the supernatant from each tube, using a 1000 µL pipet and dispose to waste. Do not disturb the pellet. Change gloves in between each sample.

8. Add 800 µL of lysis buffer using a 1000 µL pipet, using a new tip for each sample. Cap the tubes and mix by vortexing on high (~1800 rpm) in 10 second pulses for a total of 60 seconds. Change gloves in between each sample.

9. Vortex each screw-cap tube briefly (low speed, 5 – 10 seconds) and transfer the entire sample volume to a 2 mL Eppendorf tube (ensure the tubes are labeled correctly during transfer). Change gloves in between each sample. Incubate the T_0 and T_i lysate tubes at room temperature for 5 minutes.

Performed by: _____ Date: _____

WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS ANTHRACIS* SPORES - RWI

10. Vortex the PMPs on high (~1800 rpm) for 30 – 60 seconds, or until they are uniformly resuspended. Keep PMPs in suspension by briefly vortexing (3 – 5 seconds) before adding to each T_0 and T_i lysate tube.

11. Uncap one tube at a time and add 600 µL of PMPs to each T_0 and T_i tube (containing 1 mL sample). Change gloves in between each sample.

12. Vortex each T_0 and T_i tube for 5 – 10 seconds at high speed. Incubate at room temperature for 5 minutes, briefly vortex, and then place on the magnetic stand with hinged-side of the tube facing toward the magnet.

13. Invert tubes 180 degrees (upside-down) turning away from you, then right side-up, then upside down toward you, then right side-up (caps up) position, allowing all PMPs to contact the magnet.

14. Check to see if any beads are in the caps and if so, repeat the tube inversion cycle again. Let the tubes sit for 5 – 10 seconds before opening. Maintain the tube layout when transferring tubes between the magnetic stand and tube rack.

15. Uncapping one tube at a time, withdraw all liquid using a 1000 µL pipet, placing the pipet tip in the bottom of the 2 mL tube. Be sure to remove all liquid without disturbing PMPs. Use a new pipet tip to remove any residual liquid, if necessary. If liquid remains in the tube cap, remove by pipetting.

16. Uncap each tube one at a time and add 360 µL of lysis buffer using a 1000 µL pipet. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack.

17. Vortex each tube for 5 – 10 seconds (low) and place back on the magnetic stand. After all tubes are in the stand, follow tube inversion cycle, as described in Step A.13.

18. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube.

Wash Steps:

19. Uncap each tube one at a time and add 360 µL of **Salt Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack. Place tube rack back on magnetic. Invert as described in step A.13. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube. This is **1st Salt Wash.**

20. Uncap each tube one at a time and add 360 µL of **Salt Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack. Place tube rack back on magnetic. Invert as described in step A.13. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube. This is **2nd Salt Wash.**

Performed by: _____ Date: _____

WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS ANTHRACIS* SPORES - RWI

21. Uncap each tube one at a time and add 500 µL of **Alcohol Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack. Place tube rack back on magnetic. Invert as described in step A.13. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube. This is **1st Alcohol Wash.**

22. Uncap each tube one at a time and add 500 µL of **Alcohol Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack. Place tube rack back on magnetic. Invert as described in step A.13. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube. This is **2nd Alcohol Wash.**

23. Uncap each tube one at a time and add 500 µL of **Alcohol Wash Solution**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack. Place tube rack back on magnetic. Invert as described in step A.13. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube. This is **3rd Alcohol Wash.**

24. Uncap each tube one at a time and add 500 µL of **70% Ethanol**. Remove tube rack off of magnetic stand. Vortex on low setting for 5 – 10 seconds, and transfer to tube rack. Place tube rack back on magnetic. Invert as described in step A.13. Remove all the liquid as described in Step A.17. Use a new tip for each T_0 and T_i tube. This is **4th Alcohol Wash.**

25. **If necessary, us**e a 200 uL pipet to remove remaining 70% ethanol, being careful to not disturb PMPs.

26. Open all T_0 and T_i tubes and air dry for 2 minutes.

27. Close tubes and transfer to heat block. Re open tubes once placed on the heat block at 80°C until the PMPs are dry (~20 minutes, or until dry). Allow all the alcohol solution to evaporate since alcohol may interfere with analysis. If residual condensation is present, do not remove, leave it in place.

Start: _____ End:_____ Temperature:_____

28. DNA elution: While they are in the heating block add 200 µL of elution buffer to each T_0 and T_i tube, and close tube. Vortex for 10 seconds and place back on heating block for 80 seconds.

29. Briefly vortex the tubes (5 – 10 seconds) taking care to prevent the liquid from entering the tube cap and let the tube sit in the heating block for 1 minute. Reduce vortex speed if liquid appears to enter the tube cap lid.

30. Repeat Step 29 four more times.

31. Remove the tubes from the heating block, place them in a tube rack in the BSC, and incubate at room temperature for at least 5 minutes.

Start: _____ End:_____

Performed by: _____ Date: _____

WORK INSTRUCTION FOR MANUAL DNA EXTRACTION AND PURIFICATION FROM *BACILLUS ANTHRACIS* SPORES - RWI

32. Briefly vortex each tube (5 – 10 seconds) on low speed and centrifuge at 2000 rpm, 4ºC for 1 minute..

33. Briefly vortex each tube and place on the magnetic stand for at least 30 seconds.

34. Collect liquid from each T_0 and T_i tube and transfer ~80-90 uL to a clean, labeled, 1.5 mL tube on ice (check tube labels to ensure the correct order). Use a new tip for each tube. Visually verify absence of PMP carryover during final transfer. If magnetic bead carryover occurred, place 1.5 mL tube on magnet, collect liquid, and transfer to a clean, labeled, 1.5 mL tube.

35. Centrifuge tubes at 14,000 rpm at 4°C for 5 minutes to pellet any particles remaining with the eluted DNA; carefully remove supernatant from all samples and transfer to a new 1.5 mL tube using a new tip for each tube.

Start: _____ End:_____

36. Store T_0 and T_i DNA extract tubes at 4°C until PCR analysis. Continue to ***WI-RV-PCR-Native Filters***.

Note: If PCR cannot be performed within 24 hours, freeze DNA extracts at -20ºC.

Labeled:_____

Date/Time:_____ Storage Temperature:_____

Storage Location:_____

Performed by: _____ Date: _____

IV. Technical Review

Performed by: _____ Date:_____

Comments:

_____

_____

_____

APPENDIX J: WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES–SPG STICKS

WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES - SPG

I. PURPOSE/SCOPE

Duplex Rapid Viability (DRV)-PCR for *B. anthracis* spores from recovered from air filters modified from the single-plex RV-PCR described in ***BACILLUS Analytical Methods 004*** published by the EPA December 2012.

II. MATERIALS/EQUIPMENT

Materials

Enter materials used into Native Filters WI-RV-PCR-5v2 – FORM A

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	Baker Thermo Forma		N/A		
Micropipette Type: L10	Rainin		N/A		
Micropipette Type: L20	Rainin		N/A		
Micropipette Type: L200	Gilson		N/A		
Micropipette Type: L1000	Rainin		N/A		
Refrigerator	Kolpak	X57533	Rees #65	2/2019	
Freezer	Kelvinator				
Centrifuge	Eppendorf	X58983	N/A	N/A	
7500 Fast	Applied Biosystems	275017115	N/A	12/2018	

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette tips
- 96-well 0.1 mL FAST plates
- Optical caps
- Biohazard bags
- Bleach
- DNase Away
- 70% Isopropanol

WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES - SPG

III. PROCEDURE

A. Prepare samples for qPCR

Note: This step must be performed in the BSC outside the PCR clean room set-up area. Prepare a fresh aliquot of PCR-grade water per sample batch to use for 1:10 dilutions and NTCs.

1. ***T0 and Ti DNA extracts*:** Label 1.5 mL tubes with the sample identifier and "10-fold dilution". Add 90 µL of PCR-grade water to the tubes.

2. Mix T0 and Ti DNA extracts by vortexing (3 – 5 seconds), spin at 14,000 rpm for 2 minutes, and transfer 10 µL of supernatant to 1.5-mL Eppendorf tubes with 90 µL of PCR-grade water, maintaining the plate layout.

   Note: No centrifugation is required if PCR analysis is conducted immediately after DNA elution.

B. Real-time PCR Analysis of DNA Extracts

1. Decontaminate the PCR workstation by treating all work surfaces with a DNA erase, bleach solution, followed by 70% Isopropanol. After decontamination, discard gloves and replace with a new clean pair.

   Note: If gloves become contaminated, they should be disposed of and fresh gloves donned. Only open one tube at a time throughout the process. At no point, should more than one tube be open. Do not allow hands (gloved or otherwise) to pass over an open tube, PCR plate, or any reagent container. All used pipet tips, gloves and tubes must be discarded in a biohazard autoclave bag.

2. Determine the number of reactions that are to be run. Prepare a sufficient volume of Master Mix to allow for one extra reaction for every ten reactions, so that there is enough Master Mix regardless of pipetting variations. *For each batch of samples, PCR Master Mix should be made for 4 PCs, 4 NTCs, 3 PNCs (Method blank) and 6 DNA extracts per sample (3 for T0 and 3 for Ti DNA extracts).* Record sample names and reaction numbers on ***WI-RV-PCR-5v2 – FORM A.***

3. In a clean PCR-preparation hood, pipet 20 µL of Master Mix into the wells of the PCR plate. Label four wells as NTC and four as PC.

4. Add 5 µL of PCR-grade water into the NTC wells.

5. Tightly seal the NTC wells with optical caps, cover all other wells of the plate using optical caps.

6. Vortex each sample briefly, then add 5 µL to each sample well. Tightly seal the sample wells with optical caps.

Performed by: _____ Date: _____

7. Vortex the PC (*B. anthracis* DNA [10 pg/µL or 50 pg/5 µL]) and add 5 µL to each of the PC wells. Tightly seal the PC wells with optical caps.

8. ***Note: This step must be performed in the BSC outside the PCR clean room set-up area (Dead Air Box).***

C. Within the Post-Amplification Lab, Load 96-well plates onto 7500 Fast.

1. Set up 7500 Fast
    a. Open the 7500 Fast Software and a new file.
        i. Configure the New Document dialog box:
            1. Assay – Select Standard Curve (Absolute Quantification)
            2. Container – Select 96 Wells Clear
            3. Template—Select Blank, or Browse for a previously saved file.
            4. Choose the Run mode—7500 FAST.
            5. Operator—Enter your name.
            6. Comments—Enter any comments pertaining to the run.
            7. Plate Name—Enter a plate name.
            8. Click Next.
        ii. Choose the Detector for this assay
            1. Choose 6-FAM-MGB from the list, or create a new one now by clicking the **New Detector** button. Multiple detectors can be selected by using the Ctrl key.
            2. Choose **Add >>** to add the detectors to the plate document.
            3. Choose ROX™ as the passive reference from the Passive Reference drop down box.
            4. Click Next.
        iii. The setup window is split into two panes. Use the layout grid to select indiviual wells, and the Setup tab of the Well Inspector pane to apply detectors and designate well assignments. As parameters are chosen in the Setup tab, they are recorded in the Table pane at the bottom of the window.
            1. Highlight the wells desired in the layout grid.
            2. Check the **Use** box next to each detector to be analyzed for each well.
            3. Click in the **Sample Name** box, and type in the name.
            4. Choose the **Task** (Standard or NTC) from the dropdown list. If the task is Standard, you must enter the concentration before proceeding to the next well. Proceed until all the wells have been assigned all of the appropriate detectors, names, task, and concentrations (standards only).
            5. Click Finish.
        iv. Set the method parameters by clicking on the Instrument tab of the Well Inspector pane. All parameters must be changed to match Table 1.
            1. To delete a stage, click on the bar separating the stages and drag to highlight the stage to be deleted. Click [Delete Step].

Performed by: _____ Date: _____

2. To add a parameter, click the step to the left of the location where you want to place the new parameter, and choose [Add Cycle], [Add Hold], or [Add Step].
3. To change cycle times and temperatures, click in the respective boxes in each stage and type in the appropriate settings.

Table 1. 7500 FAST Method Parameters.

Temperature (°C)	Time	Cycles
95.0	*0:20*	1
95.0	*0:03*	45
60.0	*0:30*	
25 µL Total Volume		

v. Save the file. Only files with the (*.sds) extension can be run.
   1. From the top, choose File, Save As.
   2. If the document is a new plate, go to drive D, Applied Biosystems, SDS, Documents, and the appropriate folder. Name the file. From the drop down, "Files of type:" choose *ABI Prism SDS Single Plate (*.sds)*.
   3. If the document is a template, follow the same procedure. This file will already have a name and the *(*.sdt*) file extension. Change the name to identify this particular run, and change the file type to *ABI Prism SDS Single Plate (*.sds)*.
   4. Close the file.

vi. Centrifuge the plate at 300 x G for about 1 – 2 minutes at room temperature or in Labnet's MPS-1000 Mini Plate Spinner.

vii. Open the file created in step C. 1. a. v., load 96-well plate into 7500 and start run.

viii. When run is complete, burn the file to a CD.

ix. Remove 96-well plate from 7500 Fast and dispose

D. Analysis

1. Open the assay with the most current version of 7500 Fast software.
   a. Analysis can be performed using automatic settings. If required to manually set threshold and baseline, from the menu bar choose Analysis, and then choose Analysis Settings. Select Manual Ct, and Manual Baseline.
   b. From the menu bar choose Analysis, then choose Analyze from the drop down menu, or click on the large green triangle icon button in the toolbar.
   c. Highlight the unknowns, standards (only one set if there is more than one), and NTCs either in the Plate Grid pane or in the Table View pane.
   d. Click on the Results tab in the Well Inspector pane to view the Amplification Plot and Standard Curve Plot.

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e. Ensure that the desired detector appears in the Detector box in both the Amplification Plot and Standard Curve Plot. If not, chose it from the drop down menus now.
f. If analyzing based on manual threshold and baseline settings,
    i. Look at the Ct values for the standards and unknowns in the Report View pane to determine the lowest Ct value. Change the value in the End (cycle) box on the right side of the plot to adjust the baseline setting to two Ct values below the lowest Ct value whole number, ignoring values to the right of the decimal. (For example, if the lowest Ct value is 22.610105, the right cursor should be placed at 20.)
    ii. Highlight the standards and NTCs only. Click on the line that represents the threshold in the amplification plot. Move the threshold so that it is above all background "noise" generated by non-amplification, and in the lowest part of the exponential phase of all the standards. Depending on the range of the standard curve, some standards at the lowest end of the curve may not amplify well; whether or not to include them will be decided by the Program Manager or Principal Investigator.
    iii. Check the values in the Report View pane. Moving the threshold occasionally causes the Ct values to change. If necessary, readjust the baseline and threshold by repeating steps 9a through b until the baseline remains at a level two Ct values below the lowest Ct value in the Report View pane and the threshold is properly set. If changes are made that would affect the software's response to the data, such as deleting a well or changing a detector, etc., the software will reset the analysis and the data will need to be reanalyzed.
    iv. Look in the Report View pane to ensure that the Ct value for all of the NTCs is Undetermined. This means that no amplification was detected in the number of cycles this analysis ran, and is considered to be a negative result. If any of the wells show amplification at this point, contact the Program Manager or Principal Investigator.
    v. From the menu bar, choose Tools and then Report Settings. Check the boxes for the data required to be printed by your project.
    vi. Click Print, Done.
g. Highlight each individual well with a Ct less than 45, and check the Multicomponent Plot to ensure there is actual amplification. Amplification is indicated by an upward curve in the line representing the reporter dye, and if a quencher is present, a downward curve in the line representing the quencher.
h. As specified by a project, print Multicomponent Plots.
i. Annotations to be made by the analyst on the printouts:
    - Initial and date (I/D) every printout.
    - Initial, date, and error or otherwise annotate all errors and comments.
    - Indicate which, if any, wells of the Standard Curve were omitted.
    - If required by the project, every Multicomponent Plot should indicate which sample number it represents, and the actual Ct value associated with the well
j. Repeat the preceding steps to this point for every detector OR each standard curve associated with this assay.
k. Attach all printouts to the worksheet. This constitutes one data package.

Performed by: _____ Date: _____

2. After the PCR run, discard sealed PCR plate.
3. Export the .csv file
    a. Go to File -> Export -> Results.
    b. Select folder location -> Native Filters
    c. Save results type as .csv.
    d. Select save.
        i. A dialog box will open.
        ii. Check the box, Apply Report Settings for Data Columns.
    e. Burn .sds and .csv files onto a CD.

IV. Data Calculations

Calculate an average CT from the replicate reactions for T0 and T9 DNA extracts of each sample. Subtract the average CT of the T9 DNA extract from the average CT of the T0 DNA extract. If there is no CT for the T0 DNA extract (i.e., the T0 is non-detect), use 45 (total number of PCR cycles used) as the CT. The change (decrease) in the average CT value from T0 to T9 (ΔCT) ≥ 9 indicates a positive result suggesting the presence of viable *B. anthracis* spores in the sample. If an incubation time longer than 9 hours was used for the RV-PCR, instead of T9, appropriate T× (incubation time) should be used. However, (ΔCT) ≥ 9 algorithm should still be used for a positive result. Depending upon the end user's requirement, sample complexity (dirtiness) and the phase of response during an event, a lower ΔCT criterion of ≥ 6 (a two log difference in DNA concentration) and a corresponding higher endpoint PCR CT of ≤ 39 could be set. A minimum of two out of three T0 PCR replicates must result in CT values ≤ 44 (in a 45-cycle PCR) to calculate the average CT. A minimum of two out of three T9 PCR replicates (or T× for other incubation time) must result in CT values ≤ 36 to calculate the average CT for a sample result to be considered positive. Negative controls (NTCs) should not yield any measurable CT values above the background level. If CT values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat analysis. In addition, field blank samples should not yield any measurable CT values. If CT values are observed as a result of a possible contamination or cross-contamination, a careful interpretation of the CT values for the sample DNA extracts and field blanks must be done to determine if the data is considered valid or if the PCR analyses must be repeated.
Print RV-PCR results and attach to this WI package, include relevant calculations and file name.

Performed by: _____ Date: _____

V. Technical Review

Performed by: _____ Date:_____

Real World Interferents WI-RV-PCR-5v2 – FORM A

DNA ASSAY—96 Well Plate Setup for Fast 7500

Project: RWI **Barcode:** _____
Target: **Ba Duplex, Chromosome (FAM-MGBNFQ) and pXO1 (VIC-MGBNFQ)**

1. Calculate the total number of reactions per plate:

   Sample wells 48 + 4 NTC wells + 4 positive controls + 5 extras = 61 total rxns/plate (Y)

2. Prepare the Master Mix by combining the following reagents in an appropriate tube according to the following calculation:
   Reagent volume (X) x total rxns/plate (Y) = Total Volume of reagent needed

Reagent	Manufacturer	Lot No.	Exp. Date	X	Y	Total Volume (µL)
2X Fast PCR Mix				12.5 µL	61	762.5
Platinum Taq DNA Polymerase				0.25 µL	61	15.25
pXO1 For. primer (25 uM)				1 µL	61	61
pXO1 Rev. primer (25 uM)				1 µL	61	61
pXO1 Probe (2 µM)				1 µL	61	61
Chro. For. primer (25 uM)				1 µL	61	61
Chro. Rev. primer (25 uM)				1 µL	61	61
Chro. Probe (2 µM)				1 µL	61	61
PCR grade water				1.25 µL	61	91.5
Total				20 µL		

3. Distribute 20 µL of Master Mix into each reaction well, as indicated in the plate layout, below. Loosely cover all wells containing Master Mix with caps.
4. Add 5 µL of PCR-grade water to each of the NTC Wells. Cap wells tightly.
5. Add 5 µL of PNC (Method Blank) to the corresponding wells and secure the caps
6. Add 5 µL of Sample to the corresponding wells and secure the caps.
7. Add 5 µL of Positive Control to the corresponding wells and secure the caps.

Positive Control prep date		Positive Control lot	

8. Centrifuge the plate using Labnet's MPS-1000 Mini Plate Spinner at room temperature, and then load the plate onto the 7500 Fast Dx.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-SPG-TELEBO-A-S01-0 T_0			1-SPG-TELEBO-A-S01-0 T_i								
B												
C												
D												
E												
F												
G												
H	PC 50 pg	PC 50 pg	PC 50 pg	PC 50 pg					NTC	NTC	NTC	NTC

Technicians	Signature	Date
Master Mix, NTC		
Samples		
Standards		
Analyst		

Reviewed By: _____ Date: _____

APPENDIX K: WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES-VCF

WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES - VCF

I. PURPOSE/SCOPE

Duplex Rapid Viability (DRV)-PCR for *B. anthracis* spores from recovered from air filters modified from the single-plex RV-PCR described in ***BACILLUS Analytical Methods 004*** published by the EPA December 2012.

II. MATERIALS/EQUIPMENT

Materials

Enter materials used into Native Filters WI-RV-PCR-5v2 – FORM A

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	Baker Thermo Forma		N/A		
Micropipette Type: L10	Rainin		N/A		
Micropipette Type: L20	Rainin		N/A		
Micropipette Type: L200	Gilson		N/A		
Micropipette Type: L1000	Rainin		N/A		
Refrigerator	Kolpak	X57533	Rees #65	2/2019	
Freezer	Kelvinator				
Centrifuge	Eppendorf	X58983	N/A	N/A	
7500 Fast	Applied Biosystems	275017115	N/A	12/2018	

N/A = Not Applicable

Other Supplies and Equipment

- Micropipette tips
- 96-well 0.1 mL FAST plates
- Optical caps
- Biohazard bags
- Bleach
- DNase Away
- 70% Isopropanol

WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES - VCF

III. PROCEDURE

A. Prepare samples for qPCR

Note: This step must be performed in the BSC outside the PCR clean room set-up area. Prepare a fresh aliquot of PCR-grade water per sample batch to use for 1:10 dilutions and NTCs.

1. ***T0 and Ti DNA extracts*:** Label 1.5 mL tubes with the sample identifier and "10-fold dilution". Add 90 µL of PCR-grade water to the tubes.

2. Mix T0 and Ti DNA extracts by vortexing (3 – 5 seconds), spin at 14,000 rpm for 2 minutes, and transfer 10 µL of supernatant to 1.5-mL Eppendorf tubes with 90 µL of PCR-grade water, maintaining the plate layout.

   Note: No centrifugation is required if PCR analysis is conducted immediately after DNA elution.

B. Real-time PCR Analysis of DNA Extracts

1. Decontaminate the PCR workstation by treating all work surfaces with a DNA erase, bleach solution, followed by 70% Isopropanol. After decontamination, discard gloves and replace with a new clean pair.

   Note: If gloves become contaminated, they should be disposed of and fresh gloves donned. Only open one tube at a time throughout the process. At no point, should more than one tube be open. Do not allow hands (gloved or otherwise) to pass over an open tube, PCR plate, or any reagent container. All used pipet tips, gloves and tubes must be discarded in a biohazard autoclave bag.

2. Determine the number of reactions that are to be run. Prepare a sufficient volume of Master Mix to allow for one extra reaction for every ten reactions, so that there is enough Master Mix regardless of pipetting variations. *For each batch of samples, PCR Master Mix should be made for 4 PCs, 4 NTCs, 3 PNCs (Method blank) and 6 DNA extracts per sample (3 for T0 and 3 for Ti DNA extracts).* Record sample names and reaction numbers on ***WI-RV-PCR-5v2 – FORM A.***

3. In a clean PCR-preparation hood, pipet 20 µL of Master Mix into the wells of the PCR plate. Label four wells as NTC and four as PC.

4. Add 5 µL of PCR-grade water into the NTC wells.

5. Tightly seal the NTC wells with optical caps, cover all other wells of the plate using optical caps.

6. Vortex each sample briefly, then add 5 µL to each sample well. Tightly seal the sample wells with optical caps.

Performed by: _____ Date: _____

WORK INSTRUCTION RV-PCR FOR *BACILLUS ANTHRACIS* SPORES - VCF

7. Vortex the PC (*B. anthracis* DNA [10 pg/µL or 50 pg/5 µL]) and add 5 µL to each of the PC wells. Tightly seal the PC wells with optical caps.

8. ***Note: This step must be performed in the BSC outside the PCR clean room set-up area (Dead Air Box).***

C. Within the Post-Amplification Lab, Load 96-well plates onto 7500 Fast.

1. Set up 7500 Fast
    a. Open the 7500 Fast Software and a new file.
        i. Configure the New Document dialog box:
            1. Assay – Select Standard Curve (Absolute Quantification)
            2. Container – Select 96 Wells Clear
            3. Template—Select Blank, or Browse for a previously saved file.
            4. Choose the Run mode—7500 FAST.
            5. Operator—Enter your name.
            6. Comments—Enter any comments pertaining to the run.
            7. Plate Name—Enter a plate name.
            8. Click Next.
        ii. Choose the Detector for this assay
            1. Choose 6-FAM-MGB from the list, or create a new one now by clicking the **New Detector** button. Multiple detectors can be selected by using the Ctrl key.
            2. Choose **Add >>** to add the detectors to the plate document.
            3. Choose ROX™ as the passive reference from the Passive Reference drop down box.
            4. Click Next.
        iii. The setup window is split into two panes. Use the layout grid to select indiviual wells, and the Setup tab of the Well Inspector pane to apply detectors and designate well assignments. As parameters are chosen in the Setup tab, they are recorded in the Table pane at the bottom of the window.
            1. Highlight the wells desired in the layout grid.
            2. Check the **Use** box next to each detector to be analyzed for each well.
            3. Click in the **Sample Name** box, and type in the name.
            4. Choose the **Task** (Standard or NTC) from the dropdown list. If the task is Standard, you must enter the concentration before proceeding to the next well. Proceed until all the wells have been assigned all of the appropriate detectors, names, task, and concentrations (standards only).
            5. Click Finish.
        iv. Set the method parameters by clicking on the Instrument tab of the Well Inspector pane. All parameters must be changed to match Table 1.
            1. To delete a stage, click on the bar separating the stages and drag to highlight the stage to be deleted. Click [Delete Step].

Performed by: _____ Date: _____

2. To add a parameter, click the step to the left of the location where you want to place the new parameter, and choose [Add Cycle], [Add Hold], or [Add Step].
3. To change cycle times and temperatures, click in the respective boxes in each stage and type in the appropriate settings.

Table 1. 7500 FAST Method Parameters.

Temperature (°C)	Time	Cycles
95.0	*0:20*	1
95.0	*0:03*	45
60.0	*0:30*	
25 µL Total Volume		

v. Save the file. Only files with the (*.sds) extension can be run.
   1. From the top, choose File, Save As.
   2. If the document is a new plate, go to drive D, Applied Biosystems, SDS, Documents, and the appropriate folder. Name the file. From the drop down, "Files of type:" choose *ABI Prism SDS Single Plate (*.sds)*.
   3. If the document is a template, follow the same procedure. This file will already have a name and the *(*.sdt*) file extension. Change the name to identify this particular run, and change the file type to *ABI Prism SDS Single Plate (*.sds)*.
   4. Close the file.

vi. Centrifuge the plate at 300 x G for about 1 – 2 minutes at room temperature or in Labnet's MPS-1000 Mini Plate Spinner.

vii. Open the file created in step C. 1. a. v., load 96-well plate into 7500 and start run.

viii. When run is complete, burn the file to a CD.

ix. Remove 96-well plate from 7500 Fast and dispose

D. Analysis

1. Open the assay with the most current version of 7500 Fast software.
   a. Analysis can be performed using automatic settings. If required to manually set threshold and baseline, from the menu bar choose Analysis, and then choose Analysis Settings. Select Manual Ct, and Manual Baseline.
   b. From the menu bar choose Analysis, then choose Analyze from the drop down menu, or click on the large green triangle icon button in the toolbar.
   c. Highlight the unknowns, standards (only one set if there is more than one), and NTCs either in the Plate Grid pane or in the Table View pane.
   d. Click on the Results tab in the Well Inspector pane to view the Amplification Plot and Standard Curve Plot.

Performed by: _____ Date: _____

e. Ensure that the desired detector appears in the Detector box in both the Amplification Plot and Standard Curve Plot. If not, chose it from the drop down menus now.
f. If analyzing based on manual threshold and baseline settings,
    i. Look at the Ct values for the standards and unknowns in the Report View pane to determine the lowest Ct value. Change the value in the End (cycle) box on the right side of the plot to adjust the baseline setting to two Ct values below the lowest Ct value whole number, ignoring values to the right of the decimal. (For example, if the lowest Ct value is 22.610105, the right cursor should be placed at 20.)
    ii. Highlight the standards and NTCs only. Click on the line that represents the threshold in the amplification plot. Move the threshold so that it is above all background "noise" generated by non-amplification, and in the lowest part of the exponential phase of all the standards. Depending on the range of the standard curve, some standards at the lowest end of the curve may not amplify well; whether or not to include them will be decided by the Program Manager or Principal Investigator.
    iii. Check the values in the Report View pane. Moving the threshold occasionally causes the Ct values to change. If necessary, readjust the baseline and threshold by repeating steps 9a through b until the baseline remains at a level two Ct values below the lowest Ct value in the Report View pane and the threshold is properly set. If changes are made that would affect the software's response to the data, such as deleting a well or changing a detector, etc., the software will reset the analysis and the data will need to be reanalyzed.
    iv. Look in the Report View pane to ensure that the Ct value for all of the NTCs is Undetermined. This means that no amplification was detected in the number of cycles this analysis ran, and is considered to be a negative result. If any of the wells show amplification at this point, contact the Program Manager or Principal Investigator.
    v. From the menu bar, choose Tools and then Report Settings. Check the boxes for the data required to be printed by your project.
    vi. Click Print, Done.
g. Highlight each individual well with a Ct less than 45, and check the Multicomponent Plot to ensure there is actual amplification. Amplification is indicated by an upward curve in the line representing the reporter dye, and if a quencher is present, a downward curve in the line representing the quencher.
h. As specified by a project, print Multicomponent Plots.
i. Annotations to be made by the analyst on the printouts:
    - Initial and date (I/D) every printout.
    - Initial, date, and error or otherwise annotate all errors and comments.
    - Indicate which, if any, wells of the Standard Curve were omitted.
    - If required by the project, every Multicomponent Plot should indicate which sample number it represents, and the actual Ct value associated with the well
j. Repeat the preceding steps to this point for every detector OR each standard curve associated with this assay.
k. Attach all printouts to the worksheet. This constitutes one data package.

Performed by: _____ Date: _____

2. After the PCR run, discard sealed PCR plate.
3. Export the .csv file
    a. Go to File -> Export -> Results.
    b. Select folder location -> Native Filters
    c. Save results type as .csv.
    d. Select save.
        i. A dialog box will open.
        ii. Check the box, Apply Report Settings for Data Columns.
    e. Burn .sds and .csv files onto a CD.

IV. Data Calculations

Calculate an average CT from the replicate reactions for T0 and T9 DNA extracts of each sample. Subtract the average CT of the T9 DNA extract from the average CT of the T0 DNA extract. If there is no CT for the T0 DNA extract (i.e., the T0 is non-detect), use 45 (total number of PCR cycles used) as the CT. The change (decrease) in the average CT value from T0 to T9 (ΔCT) ≥ 9 indicates a positive result suggesting the presence of viable *B. anthracis* spores in the sample. If an incubation time longer than 9 hours was used for the RV-PCR, instead of T9, appropriate T× (incubation time) should be used. However, (ΔCT) ≥ 9 algorithm should still be used for a positive result. Depending upon the end user's requirement, sample complexity (dirtiness) and the phase of response during an event, a lower ΔCT criterion of ≥ 6 (a two log difference in DNA concentration) and a corresponding higher endpoint PCR CT of ≤ 39 could be set. A minimum of two out of three T0 PCR replicates must result in CT values ≤ 44 (in a 45-cycle PCR) to calculate the average CT. A minimum of two out of three T9 PCR replicates (or T× for other incubation time) must result in CT values ≤ 36 to calculate the average CT for a sample result to be considered positive. Negative controls (NTCs) should not yield any measurable CT values above the background level. If CT values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat analysis. In addition, field blank samples should not yield any measurable CT values. If CT values are observed as a result of a possible contamination or cross-contamination, a careful interpretation of the CT values for the sample DNA extracts and field blanks must be done to determine if the data is considered valid or if the PCR analyses must be repeated.
Print RV-PCR results and attach to this WI package, include relevant calculations and file name.

Performed by: _____ Date: _____

V. Technical Review

Performed by: _____ Date:_____

Real World Interferents WI-RV-PCR-5v2 – FORM A

DNA ASSAY—96 Well Plate Setup for Fast 7500

Project: RWI **Barcode:** _____
Target: **Ba Duplex, Chromosome (FAM-MGBNFQ) and pXO1 (VIC-MGBNFQ)**

1. Calculate the total number of reactions per plate:

   Sample wells 48 + 4 NTC wells + 4 positive controls + 5 extras = 61 total rxns/plate (Y)

2. Prepare the Master Mix by combining the following reagents in an appropriate tube according to the following calculation:
   Reagent volume (X) x total rxns/plate (Y) = Total Volume of reagent needed

Reagent	Manufacturer	Lot No.	Exp. Date	X	Y	Total Volume (µL)
2X Fast PCR Mix				12.5 µL	61	762.5
Platinum Taq DNA Polymerase				0.25 µL	61	15.25
pXO1 For. primer (25 uM)				1 µL	61	61
pXO1 Rev. primer (25 uM)				1 µL	61	61
pXO1 Probe (2 µM)				1 µL	61	61
Chro. For. primer (25 uM)				1 µL	61	61
Chro. Rev. primer (25 uM)				1 µL	61	61
Chro. Probe (2 µM)				1 µL	61	61
PCR grade water				1.25 µL	61	91.5
Total				20 µL		

3. Distribute 20 µL of Master Mix into each reaction well, as indicated in the plate layout, below. Loosely cover all wells containing Master Mix with caps.
4. Add 5 µL of PCR-grade water to each of the NTC Wells. Cap wells tightly.
5. Add 5 µL of PNC (Method Blank) to the corresponding wells and secure the caps
6. Add 5 µL of Sample to the corresponding wells and secure the caps.
7. Add 5 µL of Positive Control to the corresponding wells and secure the caps.

Positive Control prep date		Positive Control lot	

8. Centrifuge the plate using Labnet's MPS-1000 Mini Plate Spinner at room temperature, and then load the plate onto the 7500 Fast Dx.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-VCF-NEW-0 T_0			1-VCF-NEW-0 T_i								
B												
C												
D												
E												
F												
G												
H	PC 50 pg	PC 50 pg	PC 50 pg	PC 50 pg					NTC	NTC	NTC	NTC

Technicians	Signature	Date
Master Mix, NTC		
Samples		
Standards		
Analyst		

Reviewed By: _____ Date: _____

APPENDIX L: WORK INSTRUCTION FOR SELECTING PRESUMPTIVE *BACILLUS ANTHRACIS* STERNE COLONIES FOR QPCR CONFIRMATION

WORK INSTRUCTION FOR SELECTING PRESUMPTIVE *BACILLUS ANTHRACIS* STERNE COLONIES FOR QPCR CONFIRMATION

I. PURPOSE/SCOPE

Select and screen *B. anthracis* Sterne colonies recovered on culture plates using qPCR following the ***BACILLUS Analytical Methods 004*** published by the EPA December 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
PCR-Grade Water	Teknova			R.T.	
1 µL loop, 10 µL loop or inoculating needles				R.T.	
1.5 or 2 mL tubes				R.T.	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company		N/A		
Heat Block	Precision	9509-003	N/A	N/A	
Thermometer		N/A	N/A	N/A	
Camera		N/A	N/A	N/A	

N/A = Not Applicable

Other Supplies and Equipment

- Bleach
- 5 mL, 10 mL, and 25mL Serological Pipettes

WORK INSTRUCTION FOR SELECTING PRESUMPTIVE *BACILLUS ANTHRACIS* STERNE COLONIES FOR QPCR CONFIRMATION

Tube #	Filter ID	Volume (mL)	Morphology (B. a. Sterne or Background)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			

Filters – Record Filter ID and Morphology for Selected Colonies

III. PROCEDURE

A. Selecting colonies

1. Pipette 100 µL of PCR-grade water into 1.5 or 2 mL tubes.
2. Select colonies. Take pictures of colonies that are selected.
3. Use 1 µL loop, 10 µL loop or inoculating needle to select the colony.
4. Immerse needle into PCR-grade water and rotate to dislodge cellular material.

Performed by: _____ Date: _____

WORK INSTRUCTION FOR SELECTING PRESUMPTIVE *BACILLUS ANTHRACIS* STERNE COLONIES FOR QPCR CONFIRMATION

5. Lyse the colony suspension for 5 minutes on a heat block at 95 ± 2 °C.

Incubation start Date/Time: _____ Initials: _____
Incubation end Date/Time: _____ Initials: _____

6. Store lysed suspension at – 20 °C for qPCR analysis.

7. Prior to qPCR analysis, thaw tubes, centrifuge @ 14,000 rpm for 2 minutes. Use supernatant for qPCR.

Performed by: _____ Date: _____

IV. Technical Review

Reviewed by: _____ Date:_____

APPENDIX M: WORK INSTRUCTION FOR ENRICHMENT FOR CULTURE NON-DETECTS–SPG STICKS

WORK INSTRUCTION FOR ENRICHMENT FOR CULTURE NON-DETECTS – SPG

I. PURPOSE/SCOPE

Enrich extracted sponge and remaining culture aliquot in TSB for *B. anthracis* Sterne detection following EPA/600/R-17/213 published by the EPA December 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
PCR-Grade Water	Teknova			R.T.	
10 µL loop or inoculating needles				R.T.	
1.5 or 2 mL tubes				R.T.	
SBA Plates	BD			2 – 8 °C	
TSB	In- House	TSB110518	5/5/19	R.T.	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company		N/A		
Incubator	Precision	9509-003			
Thermometer		N/A	N/A	N/A	
Heat Block	VWR				
Refrigerator	Fisher	C3274822	115	3/2019	

N/A = Not Applicable

Other Supplies and Equipment

- 25mL Serological Pipettes

III. PROCEDURE

A. Selecting colonies

1. Add 25 mL of TSB to each specimen cup containing the extracted sponge (WI #2 Step 8) and remaining ~1 mL from the culture plating (WI # 4 Step 5) for samples that were culture non-detects.
2. Incubate cups at 37 °C ± 2 °C for 24-48 hours.

   Incubation start Date/Time: _____ Initials: _____

   Incubation end Date/Time: _____ Initials: _____

WORK INSTRUCTION FOR ENRICHMENT FOR CULTURE NON-DETECTS – SPG

3. Evaluate the TSB Enrichment.
   a. If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.
   b. If broth is turbid, record as positive growth (G+) and proceed to Step 4.

Sample Number	Filter ID	Growth (G+) or No Growth (NG)		Recorded by:
		24 hours	48 hours	
1	1-SPG-FLDBLK-A-S15-0			
2	2-SPG-STGRAT-A-S15-0			
4	4-SPG-STGRAT-A-S16-30			
6	6-SPG-STGRAT-A-S17-300			
8	8-SPG-STGRAT-A-S18-3000			
9	9-SPG-GRNBEN-A-S15-0			
10	10-SPG-WLTILE-B-S19-0			

4. Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat two times for a total of three SBA isolation plates. Store enriched samples at 2 – 8 °C.
5. Incubate the isolation plates and TSB with growth at 37 °C ± 2 °C for a maximum of three days.

   Incubation start Date/Time: _____ Initials: _____

   Incubation end Date/Time: _____ Initials: _____
6. Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (WI #6 Colony Screen).

Sample Number	Filter ID	B. a. Sterne Colonies present (Yes/No)	Number of colonies screened	Recorded by:
1	1-SPG-FLDBLK-A-S15-0			
2	2-SPG-STGRAT-A-S15-0			
4	4-SPG-STGRAT-A-S16-30			
6	6-SPG-STGRAT-A-S17-300			
8	8-SPG-STGRAT-A-S18-3000			
9	9-SPG-GRNBEN-A-S15-0			
10	10-SPG-WLTILE-B-S19-0			

7. If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section B).

B. PCR Confirmation of TSB Enriched Samples

1. Transfer 50 µL of broth with growth to a microcentrifuge tube.
2. Centrifuge at 12,000 × g for 2 minutes.

WORK INSTRUCTION FOR ENRICHMENT FOR CULTURE NON-DETECTS – SPG

3. Remove and discard the supernatant in an autoclavable biohazard container. Add 100 µL of PCR-grade water to the tube containing the bacterial pellet.
4. Resuspend the pellet by flicking the tube.
5. Lyse the suspension for 5 minutes on a heat block at 95 ± 2 °C.

   Incubation start Date/Time: _____ Initials: _____

   Incubation end Date/Time: _____ Initials: _____
6. Store lysed suspension at – 20 °C for qPCR analysis or refrigerator if processed same day.
7. Prior to qPCR analysis, thaw tubes, centrifuge @ 14,000 rpm for 2 minutes. Use supernatant for qPCR.

Performed by: _____ Date: _____

IV. Technical Review

Reviewed by: _____ Date:_____

APPENDIX N: WORK INSTRUCTION
TSB ENRICHMENT FOR CULTURE–VCF

WORK INSTRUCTION TSB ENRICHMENT FOR CULTURE - VCF

I. PURPOSE/SCOPE

Enrich extracted sponge and remaining culture aliquot in TSB for *B. anthracis* Sterne detection following EPA/600/R-17/213 published by the EPA December 2017.

II. MATERIALS/EQUIPMENT

Materials

Item	Manufacturer	Lot Number	Exp. Date	Storage Temp.	Initials & Date
PCR-Grade Water	Teknova			R.T.	
10 µL loop or inoculating needles				R.T.	
1.5 or 2 mL tubes				R.T.	
SBA Plates	BD			2 – 8 °C	
TSB	In- House			2 – 8 °C	

N/A = Not Applicable

Equipment

Item	Manufacturer	Serial Number	Thermometer/ Rees #	Calibration Due	Initials & Date
Biosafety Cabinet (BSC)	The Baker Company		N/A		
Incubator	Precision	9509-003			
Thermometer		N/A	N/A	N/A	
Heat Block	VWR				
Refrigerator	Fisher	C3274822	115	3/2019	

N/A = Not Applicable

Other Supplies and Equipment

- 25mL Serological Pipettes

III. PROCEDURE

A. Selecting colonies

1. Add 30 mL of TSB to each 2 oz. cup containing the cassette filter (WI #2 Step 13).
2. Incubate cups at 37 °C ± 2 °C for 24-48 hours.

   Incubation start Date/Time: _____ Initials: _____

   Incubation end Date/Time: _____ Initials: _____
3. Evaluate the TSB Enrichment.

WORK INSTRUCTION TSB ENRICHMENT FOR CULTURE - VCF

a. If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.

b. If broth is turbid, record as positive growth (G+) and proceed to Step 4.

Sample Number	Filter ID	Growth (G+) or No Growth (NG)		Recorded by:
		24 hours	48 hours	
1	1-VCF-FLCON-B-S15-0			
2	2-VCF-STEPS-B-S15-0			
3	3-VCF-FLCON-B-S16-30			
4	4-VCF-STEPS-B-S16-30			
5	5-VCF-FLCON-B-S17-300			
6	6-VCF-STEPS-B-S17-300			
7	7-VCF-FLCON-B-S18-3,000			
8	8-VCF-STEPS-B-S18-3,000			
9	9-VCF-SWCON-A-S15-0			
10	10-VCF-PAVEMT-A-S15-0			
11	11-VCF-SWCON-A-S16-30			
12	12-VCF-PAVEMT-A-S16-30			
13	13-VCF-SWCON-A-S17-300			
14	14-VCF-PAVEMT-A-S17-300			
15	15-VCF-SWCON-A-S18-3,000			
16	16-VCF-PAVEMT-A-S18-3,000			

4. Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat two times for a total of three SBA isolation plates. Store enriched samples at 2 – 8 °C.

5. Incubate the isolation plates and TSB with growth at 37 °C ± 2 °C for a maximum of three days.

Incubation start Date/Time: _____ Initials: _____

Incubation end Date/Time: _____ Initials: _____

6. Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (WI #6 Colony Screen).

Sample Number	Filter ID	B. a. Sterne Colonies present (Yes/No)	Number of colonies screened	Recorded by:
1	1-VCF-FLCON-B-S15-0			
2	2-VCF-STEPS-B-S15-0			
3	3-VCF-FLCON-B-S16-30			
4	4-VCF-STEPS-B-S16-30			
5	5-VCF-FLCON-B-S17-300			

WORK INSTRUCTION TSB ENRICHMENT FOR CULTURE - VCF

Sample Number	Filter ID	B. a. Sterne Colonies present (Yes/No)	Number of colonies screened	Recorded by:
6	6-VCF-STEPS-B-S17-300			
7	7-VCF-FLCON-B-S18-3,000			
8	8-VCF-STEPS-B-S18-3,000			
9	9-VCF-SWCON-A-S15-0			
10	10-VCF-PAVEMT-A-S15-0			
11	11-VCF-SWCON-A-S16-30			
12	12-VCF-PAVEMT-A-S16-30			
13	13-VCF-SWCON-A-S17-300			
14	14-VCF-PAVEMT-A-S17-300			
15	15-VCF-SWCON-A-S18-3,000			
16	16-VCF-PAVEMT-A-S18-3,000			

7. If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section B).

B. PCR Confirmation of TSB Enriched Samples

1. Transfer 50 µL of broth with growth to a microcentrifuge tube.

2. Centrifuge at 12,000 × g for 2 minutes.

3. Remove and discard the supernatant in an autoclavable biohazard container. Add 100 µL of PCR-grade water to the tube containing the bacterial pellet.

4. Resuspend the pellet by flicking the tube.

5. Lyse the suspension for 5 minutes on a heat block at 95 ± 2 °C.

Incubation start Date/Time: _____ Initials: _____

Incubation end Date/Time: _____ Initials: _____

6. Store lysed suspension at – 20 °C for qPCR analysis or refrigerator if processed same day.

7. Prior to qPCR analysis, thaw tubes, centrifuge @ 14,000 rpm for 2 minutes. Use supernatant for qPCR.

Performed by: _____ Date: _____

IV. Technical Review

Reviewed by: _____ Date:_____

APPENDIX O: CULTURE RESULTS FOR SPONGE-STICK SAMPLES USING SHEEP BLOOD AGAR MEDIUM

Sponge-Stick Floor Tile

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
10-SPG-FLTILE-B-S01-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
1-SPG-FLTILE-B-S05-0	0.0E+00	25.8	2	0	0.0	0	#DIV/0!
	0.0E+00	25.8	8	0	0.0	0	#DIV/0!
1-SPG-FLTILE-B-S09-0	0.0E+00	26	2	0	0.0	0	#DIV/0!
	0.0E+00	26	8	0	0.0	0	#DIV/0!
12-SPG-FLTILE-B-S02-30	3.1E+01	25	2	5	2.5	63	201.6
	3.1E+01	25	8	0	0.0	0	0.0
3-SPG-FLTILE-B-S06-30	2.3E+01	25	2	0	0.0	0	0.0
	2.3E+01	25	8	0	0.0	0	0.0
2-SPG-FLTILE-B-S10-30	2.4E+01	24.8	2	1	0.5	12	51.7
	2.4E+01	24.8	8	0	0.0	0	0.0
3-SPG-FLTILE-B-S11-30	2.4E+01	24.6	2	1	0.5	12	51.3
	2.4E+01	24.6	8	0	0.0	0	0.0
4-SPG-FLTILE-B-S16-30	3.6E+01	25	2	0	0.0	0	0.0
	3.6E+01	25	8	0	0.0	0	0.0
14-SPG-FLTILE-B-S03-300	3.1E+02	25	2	1	0.5	13	4.0
	3.1E+02	25	8	4	0.5	13	4.0
5-SPG-FLTILE-B-S07-300	2.3E+02	24.8	2	8	4.0	99	43.1
	2.3E+02	24.8	8	0	0.0	0	0.0
4-SPG-FLTILE-B-S12-300	2.4E+02	24.2	2	0	0.0	0	0.0
	2.4E+02	24.2	8	0	0.0	0	0.0
5-SPG-FLTILE-B-S13-300	2.4E+02	26.4	2	1	0.5	13	5.5
	2.4E+02	26.4	8	0	0.0	0	0.0
13-SPG-FLTILE-B-S17-300	3.6E+02	25	2	16	8.0	200	55.6
	3.6E+02	25	8	12	1.5	38	10.4
16-SPG-FLTILE-B-S04-3000	3.1E+03	25	2	77	38.5	963	31.0
	3.1E+03	25	8	156	19.5	488	15.7
7-SPG-FLTILE-B-S08-3000	2.3E+03	24.8	2	40	20.0	496	21.6
	2.3E+03	24.8	8	0	0.0	0	0.0
6-SPG-FLTILE-B-S14-3000	2.4E+03	26.2	2	50	25.0	655	27.3
	2.4E+03	26.2	8	38	4.8	124	5.2
7-SPG-FLTILE-B-S15-3000	2.4E+03	25.6	2	28	14.0	358	14.9
	2.4E+03	25.6	8	42	5.3	134	5.6
16-SPG-FLTILE-B-S18-3000	3.6E+03	26.8	2	37	18.5	496	13.8
	3.6E+03	26.8	8	112	14.0	375	10.4

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Floor Concrete

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
2-SPG-FLCON-B-S01-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
2-SPG-FLCON-B-S05-0	0.0E+00	25.2	2	0	0.0	0	#DIV/0!
	0.0E+00	25.2	8	0	0.0	0	#DIV/0!
1-SPG-FLCON-B-S15-0	0.0E+00	24.8	2	0	0.0	0	#DIV/0!
	0.0E+00	24.8	8	0	0.0	0	#DIV/0!
4-SPG-FLCON-B-S02-30	3.1E+01	25	2	3	1.5	38	121.0
	3.1E+01	25	8	0	0.0	0	0.0
4-SPG-FLCON-B-S06-30	2.3E+01	25	2	0	0.0	0	0.0
	2.3E+01	25	8	0	0.0	0	0.0
9-SPG-FLCON-B-S09-30	2.4E+01	24.8	2	1	0.5	12	51.7
	2.4E+01	24.8	8	0	0.0	0	0.0
10-SPG-FLCON-B-S10-30	2.4E+01	23.8	2	1	0.5	12	49.6
	2.4E+01	23.8	8	0	0.0	0	0.0
3-SPG-FLCON-B-S16-30	3.4E+01	24	2	0	0.0	0	0.0
	3.4E+01	24	8	0	0.0	0	0.0
6-SPG-FLCON-B-S03-300	3.1E+02	25	2	3	1.5	38	12.1
	3.1E+02	25	8	0	0.0	0	0.0
6-SPG-FLCON-B-S07-300	2.3E+02	24	2	5	2.5	60	26.1
	2.3E+02	24	8	0	0.0	0	0.0
13-SPG-FLCON-B-S11-300	2.4E+02	24.8	2	2	1.0	25	10.3
	2.4E+02	24.8	8	0	0.0	0	0.0
14-SPG-FLCON-B-S12-300	2.4E+02	24	2	6	3.0	72	30.0
	2.4E+02	24	8	1	0.1	3	1.3
5-SPG-FLCON-B-S17-300	3.4E+02	25	2	4	2.0	50	14.7
	3.4E+02	25	8	12	1.5	38	11.0
8-SPG-FLCON-B-S04-3000	3.1E+03	25	2	71	35.5	888	28.6
	3.1E+03	25	8	140	17.5	438	14.1
8-SPG-FLCON-B-S08-3000	2.3E+03	25.4	2	53	26.5	673	29.3
	2.3E+03	25.4	8	0	0.0	0	0.0
15-SPG-FLCON-B-S13-3000	2.4E+03	23.4	2	50	25.0	585	24.4
	2.4E+03	23.4	8	0	0.0	0	0.0
16-SPG-FLCON-B-S14-3000	2.4E+03	23.4	2	48	24.0	562	23.4
	2.4E+03	23.4	8	81	10.1	237	9.9
7-SPG-FLCON-B-S18-3000	3.4E+03	24.2	2	43	21.5	520	15.3
	3.4E+03	24.2	8	112	14.0	339	10.0

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Steps (with Metal Grid)

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-SPG-STEPS-B-S01-0	0.0E+00	23	2	0	0.0	0	#DIV/0!
	0.0E+00	23	8	0	0.0	0	#DIV/0!
9-SPG-STEPS-B-S05-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
2-SPG-STEPS-B-S15-0	0.0E+00	25.4	2	0	0.0	0	#DIV/0!
	0.0E+00	25.4	8	0	0.0	0	#DIV/0!
3-SPG-STEPS-B-S02-30	1.9E+00	25.1	2	0	0.0	0	0.0
	1.9E+00	25.1	8	0	0.0	0	0.0
11-SPG-STEPS-B-S06-30	2.3E+01	25.5	2	0	0.0	0	0.0
	2.3E+01	25.5	8	0	0.0	0	0.0
11-SPG-STEPS-B-S09-30	2.4E+01	23.8	2	0	0.0	0	0.0
	2.4E+01	23.8	8	0	0.0	0	0.0
3-SPG-STEPS-B-S11-30	3.8E+01	25.1	2	0	0.0	0	0.0
	3.8E+01	25.1	8	0	0.0	0	0.0
4-SPG-STEPS-B-S16-30	3.4E+01	24.2	2	15	7.5	182	533.8
	3.4E+01	24.2	8	74	9.3	224	658.4
5-SPG-STEPS-B-S03-300	1.9E+02	24	2	0	0.0	0	0.0
	1.9E+02	24	8	0	0.0	0	0.0
13-SPG-STEPS-B-S07-300	2.3E+02	25	2	0	0.0	0	0.0
	2.3E+02	25	8	0	0.0	0	0.0
12-SPG-STEPS-B-S10-300	2.4E+02	24.8	2	8	4.0	99	41.3
	2.4E+02	24.8	8	0	0.0	0	0.0
4-SPG-STEPS-B-S12-300	3.8E+02	26	2	0	0.0	0	0.0
	3.8E+02	26	8	0	0.0	0	0.0
6-SPG-STEPS-B-S17-300	3.4E+02	25	2	0	0.0	0	0.0
	3.4E+02	25	8	0	0.0	0	0.0
7-SPG-STEPS-B-S04-3000	1.9E+03	27.4	2	46	23.0	630	33.2
	1.9E+03	27.4	8	59	7.4	202	10.6
15-SPG-STEPS-B-S08-3000	2.3E+03	24	2	18	9.0	216	9.4
	2.3E+03	24	8	0	0.0	0	0.0
15-SPG-STEPS-B-S13-3000	3.8E+03	23	2	43	21.5	495	13.0
	3.8E+03	23	8	14	1.8	40	1.1
16-SPG-STEPS-B-S14-3000	3.8E+03	25.4	2	16	8.0	203	5.3
	3.8E+03	25.4	8	31	3.9	98	2.6
8-SPG-STEPS-B-S18-3000	3.4E+03	24.4	2	36	18.0	439	12.9
	3.4E+03	24.4	8	120	15.0	366	10.8

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Wall Tile

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
10-SPNG-WLTILE-B-S01-0	0.0E+00	22	2	0	0.0	0	#DIV/0!
	0.0E+00	22	8	0	0.0	0	#DIV/0!
10-SPG-WLTILE-B-S05-0	0.0E+00	25.5	2	0	0.0	0	#DIV/0!
	0.0E+00	25.5	8	0	0.0	0	#DIV/0!
1-SPG-WLTILE-B-S15-0	0.0E+00	24.8	2	0	0.0	0	#DIV/0!
	0.0E+00	24.8	8	0	0.0	0	#DIV/0!
10-SPG-WLTILE-B-S19-0	0.0E+00	24.3	2	0	0.0	0	#DIV/0!
	0.0E+00	24.3	8	0	0.0	0	#DIV/0!
12-SPNG-WLTILE-B-S02-30	2.1E+01	22	2	3	1.5	33	157.1
	2.1E+01	22	8	6	0.8	17	78.6
12-SPG-WLTILE-B-S06-30	2.3E+01	24.5	2	0	0.0	0	0.0
	2.3E+01	24.5	8	0	0.0	0	0.0
10-SPG-WLTILE-B-S10-30	3.8E+01	24.2	2	2	1.0	24	63.7
	3.8E+01	24.2	8	7	0.9	21	55.7
9-SPG-WLTILE-B-S09-30	3.8E+01	23.8	2	0	0.0	0	0.0
	3.8E+01	23.8	8	2	0.3	6	15.7
3-SPG-WLTILE-B-S16-30	2.4E+01	22.4	2	0	0.0	0	0.0
	2.4E+01	22.4	8	3	0.4	8	35.0
14-SPNG-WLTILE-B-S03-300	2.1E+02	22	2	10	5.0	110	52.4
	2.1E+02	22	8	22	2.8	61	28.8
14-SPG-WLTILE-B-S07-300	2.3E+02	26	2	6	3.0	78	33.9
	2.3E+02	26	8	21	2.6	68	29.7
11-SPG-WLTILE-B-S11-300	3.8E+02	28	2	9	4.5	126	33.2
	3.8E+02	28	8	37	4.6	130	34.1
12-SPG-WLTILE-B-S12-300	3.8E+02	25.8	2	7	3.5	90	23.8
	3.8E+02	25.8	8	18	2.3	58	15.3
5-SPG-WLTILE-B-S17-300	2.4E+02	25.6	2	5	2.5	64	26.7
	2.4E+02	25.6	8	4	0.5	13	5.3
16-SPNG-WLTILE-B-S04-3000	2.1E+03	22	2	83	41.5	913	43.5
	2.1E+03	22	8	216	27.0	594	28.3
16-SPG-WLTILE-B-S08-3000	2.3E+03	25.5	2	55	27.5	701	30.5
	2.3E+03	25.5	8	115	14.4	367	15.9
13-SPG-WLTILE-B-S13-3000	3.8E+03	25	2	66	33.0	825	21.7
	3.8E+03	25	8	160	20.0	500	13.2
14-SPG-WLTILE-B-S14-3000	3.8E+03	23.6	2	10	5.0	118	3.1
	3.8E+03	23.6	8	172	21.5	507	13.4
7-SPG-WLTILE-B-S18-3000	2.4E+03	25	2	26	13.0	325	13.5
	2.4E+03	25	8	33	4.1	103	4.3
Use values highlighted in green for reporting.							
Previously called UD for undetected 3/27/2019 scn.							

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Glass Window

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-SPG-GLSWIN-B-S01-0	0.0E+00	26.8	2	0	0.0	0	#DIV/0!
	0.0E+00	26.8	8	0	0.0	0	#DIV/0!
1-SPG-GLSWIN-B-S05-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
1-SPG-GLSWIN-B-S13-0	0.0E+00	24.2	2	0	0.0	0	#DIV/0!
	0.0E+00	24.2	8	0	0.0	0	#DIV/0!
3-SPG-GLSWIN-B-S02-30	1.8E+01	24.4	2	1	0.5	12	67.8
	1.8E+01	24.4	8	2	0.3	6	33.9
2-SPG-GLSWIN-B-S06-30	3.4E+01	25	2	0	0.0	0	0.0
	3.4E+01	25	8	3	0.4	9	27.6
3-SPG-GLSWIN-B-S07-30	3.4E+01	25	2	1	0.5	13	36.8
	3.4E+01	25	8	0	0.0	0	0.0
4-SPG-GLSWIN-B-S08-30	3.4E+01	24.6	2	2	1.0	25	72.4
	3.4E+01	24.6	8	2	0.3	6	18.1
2-SPG-GLSWIN-B-S14-30	3.8E+01	24.8	2	2	1.0	25	65.3
	3.8E+01	24.8	8	0	0.0	0	0.0
5-SPG-GLSWIN-B-S03-300	1.8E+02	24.4	2	8	4.0	98	54.2
	1.8E+02	24.4	8	16	2.0	49	27.1
5-SPG-GLSWIN-B-S09-300	3.4E+02	23.4	2	5	2.5	59	17.2
	3.4E+02	23.4	8	27	3.4	79	23.2
6-SPG-GLSWIN-B-S10-300	3.4E+02	22.4	2	9	4.5	101	29.6
	3.4E+02	22.4	8	26	3.3	73	21.4
5-SPG-GLSWIN-B-S15-300	3.8E+02	24	2	12	6.0	144	37.9
	3.8E+02	24	8	37	4.6	111	29.2
6-SPG-GLSWIN-B-S16-300	3.8E+02	25	2	10	5.0	125	32.9
	3.8E+02	25	8	43	5.4	134	35.4
7-SPG-GLSWIN-B-S04-3000	1.8E+03	25.2	2	46	23.0	580	32.2
	1.8E+03	25.2	8	147	18.4	463	25.7
7-SPG-GLSWIN-B-S11-3000	3.4E+03	24.6	2	47	23.5	578	17.0
	3.4E+03	24.6	8	102	12.8	314	9.2
8-SPG-GLSWIN-B-S12-3000	3.4E+03	26.8	2	49	24.5	657	19.3
	3.4E+03	26.8	8	98	12.3	328	9.7
7-SPG-GLSWIN-B-S17-3000	3.8E+03	25	2	52	26.0	650	17.1
	3.8E+03	25	8	0	0.0	0	0.0
8-SPG-GLSWIN-B-S18-3000	3.8E+03	24.4	2	59	29.5	720	18.9
	3.8E+03	24.4	8	178	22.3	543	14.3

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Electronic Display Panel, Below Ground

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
2-SPG-EDPAN(B)-B-S01-0	0.0E+00	30	2	0	0.0	0	#DIV/0!
	0.0E+00	30	8	0	0.0	0	#DIV/0!
9-SPG-EDPAN(B)-B-S05-0	0.0E+00	25.8	2	0	0.0	0	#DIV/0!
	0.0E+00	25.8	8	0	0.0	0	#DIV/0!
9-SPG-EDPAN(B)-B-S13-0	0.0E+00	23.3	2	0	0.0	0	#DIV/0!
	0.0E+00	23.3	8	0	0.0	0	#DIV/0!
4-SPG-EDPAN(B)-B-S02-30	1.9E+00	25.2	2	0	0.0	0	0.0
	1.9E+00	25.2	8	0	0.0	0	0.0
10-SPG-EDPAN(B)-B-S06-30	3.4E+01	25	2	0	0.0	0	0.0
	3.4E+01	25	8	4	0.5	13	36.8
11-SPG-EDPAN(B)-B-S07-30	3.4E+01	23.4	2	2	1.0	23	68.8
	3.4E+01	23.4	8	0	0.0	0	0.0
12-SPG-EDPAN(B)-B-S08-30	3.4E+01	24.6	2	0	0.0	0	0.0
	3.4E+01	24.6	8	0	0.0	0	0.0
10-SPG-EDPAN(B)-B-S14-30	3.6E+01	24	2	1	0.5	12	33.3
	3.6E+01	24	8	0	0.0	0	0.0
6-SPG-EDPAN(B)-B-S03-300	1.9E+02	24.8	2	5	2.5	62	32.6
	1.9E+02	24.8	8	14	1.8	43	22.8
13-SPG-EDPAN(B)-B-S09-300	3.4E+02	24.3	2	6	3.0	73	21.4
	3.4E+02	24.3	8	22	2.8	67	19.7
14-SPG-EDPAN(B)-B-S10-300	3.4E+02	24.8	2	4	2.0	50	14.6
	3.4E+02	24.8	8	17	2.1	53	15.5
11-SPG-EDPAN(B)-B-S15-300	3.6E+02	24.8	2	13	6.5	161	44.8
	3.6E+02	24.8	8	27	3.4	84	23.3
12-SPG-EDPAN(B)-B-S16-300	3.6E+02	24.8	2	12	6.0	149	41.3
	3.6E+02	24.8	8	33	4.1	102	28.4
8-SPG-EDPAN(B)-B-S04-3000	1.9E+03	26.4	2	61	30.5	805	42.4
	1.9E+03	26.4	8	172	21.5	568	29.9
15-SPG-EDPAN(B)-B-S11-3000	3.4E+03	23	2	68	34.0	782	23.0
	3.4E+03	23	8	132	16.5	380	11.2
16-SPG-EDPAN(B)-B-S12-3000	3.4E+03	24.2	2	50	25.0	605	17.8
	3.4E+03	24.2	8	156	19.5	472	13.9
14-SPG-EDPAN(B)-B-S17-3000	3.6E+03	25	2	48	24.0	600	16.7
	3.6E+03	25	8	134	16.8	419	11.6
15-SPG-EDPAN(B)-B-S18-3000	3.6E+03	24.4	2	62	31.0	756	21.0
	3.6E+03	24.4	8	135	16.9	412	11.4

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Glass Panel

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
9-SPNG-GLSPAN-B-S01-0	0.0E+00	22	2	0	0.0	0	#DIV/0!
	0.0E+00	22	8	0	0.0	0	#DIV/0!
1-SPG-GLSPAN-B-S05-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
2-SPG-GLSPNL-B-S13-0	0.0E+00	24.4	2	0	0.0	0	#DIV/0!
	0.0E+00	24.4	8	0	0.0	0	#DIV/0!
11-SPNG-GLSPAN-B-S02-30	2.1E+01	22	2	2	1.0	22	104.8
	2.1E+01	22	8	5	0.6	14	65.5
2-SPG-GLSPAN-B-S06-30	4.8E+01	23.4	2	0	0.0	0	0.0
	4.8E+01	23.4	8	7	0.9	20	43.0
3-SPG-GLSPAN-B-S07-30	4.8E+01	24	2	1	0.5	12	25.2
	4.8E+01	24	8	2	0.3	6	12.6
4-SPG-GLSPAN-B-S08-30	4.8E+01	24.6	2	2	1.0	25	51.7
	4.8E+01	24.6	8	10	1.3	31	64.6
3-SPG-GLSPNL-B-S14-30	3.6E+01	26.8	2	1	0.5	13	37.2
	3.6E+01	26.8	8	2	0.3	7	18.6
13-SPNG-GLSPAN-B-S03-300	2.1E+02	22	2	6	3.0	66	31.4
	2.1E+02	22	8	38	4.8	105	49.8
5-SPG-GLSPAN-B-S09-300	4.8E+02	25	2	21	10.5	263	55.1
	4.8E+02	25	8	43	5.4	134	28.2
6-SPG-GLSPAN-B-S10-300	4.8E+02	23.8	2	14	7.0	167	35.0
	4.8E+02	23.8	8	54	6.8	161	33.8
5-SPG-GLSPNL-B-S15-300	3.6E+02	25.8	2	7	3.5	90	25.1
	3.6E+02	25.8	8	24	3.0	77	21.5
6-SPG-GLSPNL-B-S16-300	3.6E+02	25.6	2	9	4.5	115	32.0
	3.6E+02	25.6	8	37	4.6	118	32.9
15-SPNG-GLSPAN-B-S04-3000	2.1E+03	22	2	76	38.0	836	39.8
	2.1E+03	22	8	222	27.8	611	29.1
7-SPG-GLSPAN-B-S11-3000	4.8E+03	24	2	115	57.5	1380	29.0
	4.8E+03	24	8	206	25.8	618	13.0
8-SPG-GLSPAN-B-S12-3000	4.8E+03	23	2	90	45.0	1035	21.7
	4.8E+03	23	8	232	29.0	667	14.0
7-SPG-GLSPNL-B-S17-3000	3.6E+03	24.6	2	82	41.0	1009	28.0
	3.6E+03	24.6	8	192	24.0	590	16.4
8-SPG-GLSPNL-B-S18-3000	3.6E+03	22	2	76	38.0	836	23.2
	3.6E+03	22	8	234	29.3	644	17.9

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Fluorescent Light Fixture

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
9-SPG-FLLFIX-B-S01-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
9-SPG-FLLFIX-B-S05-0	0.0E+00	26	2	0	0.0	0	#DIV/0!
	0.0E+00	26	8	0	0.0	0	#DIV/0!
1-SPG-FLLFIX-B-S13-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
11-SPG-FLLFIX-B-S02-30	3.1E+01	25	2	2	1.0	25	80.6
	3.1E+01	25	8	2	0.3	6	20.2
10-SPG-FLLFIX-B-S06-30	4.8E+01	24.6	2	2	1.0	25	51.7
	4.8E+01	24.6	8	3	0.4	9	19.4
11-SPG-FLLFIX-B-S07-30	4.8E+01	23.6	2	3	1.5	35	74.4
	4.8E+01	23.6	8	5	0.6	15	31.0
12-SPG-FLLFIX-B-S08-30	4.8E+01	24.6	2	4	2.0	49	103.4
	4.8E+01	24.6	8	3	0.4	9	19.4
2-SPG-FLLFIX-B-S14-30	2.6E+01	25	2	0	0.0	0	0.0
	2.6E+01	25	8	0	0.0	0	0.0
13-SPG-FLLFIX-B-S03-300	3.1E+02	25	2	7	3.5	88	28.2
	3.1E+02	25	8	14	1.8	44	14.1
13-SPG-FLLFIX-B-S09-300	4.8E+02	25.6	2	8	4.0	102	21.5
	4.8E+02	25.6	8	23	2.9	74	15.5
14-SPG-FLLFIX-B-S10-300	4.8E+02	24.8	2	12	6.0	149	31.3
	4.8E+02	24.8	8	41	5.1	127	26.7
3-SPG-FLLFIX-B-S15-300	2.6E+02	24.2	2	8	4.0	97	37.2
	2.6E+02	24.2	8	25	3.1	76	29.1
4-SPG-FLLFIX-B-S16-300	2.6E+02	24.2	2	9	4.5	109	41.9
	2.6E+02	24.2	8	19	2.4	57	22.1
15-SPG-FLLFIX-B-S04-3000	3.1E+03	25	2	60	30.0	750	24.2
	3.1E+03	25	8	224	28.0	700	22.6
15-SPG-FLLFIX-B-S11-3000	4.8E+03	24.2	2	96	48.0	1162	24.4
	4.8E+03	24.2	8	196	24.5	593	12.5
16-SPG-FLLFIX-B-S12-3000	4.8E+03	24	2	77	38.5	924	19.4
	4.8E+03	24	8	0	0.0	0	0.0
6-SPG-FLLFIX-B-S17-3000	2.6E+03	25	2	55	27.5	688	26.4
	2.6E+03	25	8	128	16.0	400	15.4
8-SPG-FLLFIX-B-S18-3000	2.6E+03	23.2	2	65	32.5	754	29.0
	2.6E+03	23.2	8	136	17.0	394	15.2

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Overhead Sign

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
9-SPG-OHSIGN-B-S01-0	0.0E+00	24.8	2	0	0.0	0	#DIV/0!
	0.0E+00	24.8	8	0	0.0	0	#DIV/0!
1-SPG-OHSIGN-B-S05-0	0.0E+00	24.2	2	0	0.0	0	#DIV/0!
	0.0E+00	24.2	8	0	0.0	0	#DIV/0!
9-SPG-OHSIGN-B-S12-0	0.0E+00	24.2	2	0	0.0	0	#DIV/0!
	0.0E+00	24.2	8	0	0.0	0	#DIV/0!
11-SPG-OHSIGN-B-S02-30	1.6E+01	24	2	0	0.0	0	0.0
	1.6E+01	24	8	2	0.3	6	37.5
2-SPG-OHSIGN-B-S06-30	1.7E+01	24.9	2	0	0.0	0	0.0
	1.7E+01	24.9	8	3	0.4	9	54.9
3-SPG-OHSIGN-B-S07-30	1.7E+01	24.6	2	2	1.0	25	144.7
	1.7E+01	24.6	8	1	0.1	3	18.1
10-SPG-OHSIGN-B-S13-30	2.6E+01	25.4	2	2	1.0	25	97.7
	2.6E+01	25.4	8	2	0.3	6	24.4
11-SPG-OHSIGN-B-S14-30	2.6E+01	24.2	2	2	1.0	24	93.1
	2.6E+01	24.2	8	2	0.3	6	23.3
13-SPG-OHSIGN-B-S03-300	1.6E+02	24	2	3	1.5	36	22.5
	1.6E+02	24	8	17	2.1	51	31.9
5-SPG-OHSIGN-B-S08-300	1.7E+02	25.8	2	4	2.0	52	30.4
	1.7E+02	25.8	8	10	1.3	32	19.0
6-SPG-OHSIGN-B-S09-300	1.7E+02	24.4	2	8	4.0	98	57.4
	1.7E+02	24.4	8	18	2.3	55	32.3
12-SPG-OHSIGN-B-S15-300	2.6E+02	24.6	2	15	7.5	185	71.0
	2.6E+02	24.6	8	29	3.6	89	34.3
13-SPG-OHSIGN-B-S16-300	2.6E+02	24.8	2	8	4.0	99	38.2
	2.6E+02	24.8	8	7	0.9	22	8.3
15-SPG-OHSIGN-B-S04-3000	1.6E+03	25	2	48	24.0	600	37.5
	1.6E+03	25	8	168	21.0	525	32.8
7-SPG-OHSIGN-B-S10-3000	1.7E+03	24.6	2	68	34.0	836	49.2
	1.7E+03	24.6	8	146	18.3	449	26.4
8-SPG-OHSIGN-B-S11-3000	1.7E+03	24.5	2	50	25.0	613	36.0
	1.7E+03	24.5	8	107	13.4	328	19.3
15-SPG-OHSIGN-B-S17-3000	2.6E+03	26.2	2	46	23.0	603	23.2
	2.6E+03	26.2	8	81	10.1	265	10.2
16-SPG-OHSIGN-B-S18-3000	2.6E+03	24.6	2	42	21.0	517	19.9
	2.6E+03	24.6	8	84	10.5	258	9.9

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Metro Card Machine

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
2-SPG-MCMACH-B-S01-0	0.0E+00	23.8	2	0	0.0	0	#DIV/0!
	0.0E+00	23.8	8	0	0.0	0	#DIV/0!
9-SPG-MCMACH-B-S05-0	0.0E+00	24.4	2	0	0.0	0	#DIV/0!
	0.0E+00	24.4	8	0	0.0	0	#DIV/0!
2-SPG-MCMACH-B-S15-0	0.0E+00	26.2	2	0	0.0	0	#DIV/0!
	0.0E+00	26.2	8	0	0.0	0	#DIV/0!
4-SPG-MCMACH-B-S02-30	1.6E+01	27.6	2	0	0.0	0	0.0
	1.6E+01	27.6	8	1	0.1	3	21.6
10-SPG-MCMACH-B-S06-30	1.7E+01	24	2	0	0.0	0	0.0
	1.7E+01	24	8	0	0.0	0	0.0
11-SPG-MCMACH-B-S07-30	1.7E+01	27	2	0	0.0	0	0.0
	1.7E+01	27	8	0	0.0	0	0.0
12-SPG-MCMACH-B-S08-30	1.7E+01	24.6	2	1	0.5	12	72.4
	1.7E+01	24.6	8	0	0.0	0	0.0
4-SPG-MCMACH-B-S16-30	2.4E+01	24.6	2	0	0.0	0	0.0
	2.4E+01	24.6	8	0	0.0	0	0.0
6-SPG-MCMACH-B-S03-300	1.6E+02	24.6	2	8	4.0	98	61.5
	1.6E+02	24.6	8	9	1.1	28	17.3
13-SPG-MCMACH-B-S09-300	1.7E+02	24.8	2	7	3.5	87	51.1
	1.7E+02	24.8	8	0	0.0	0	0.0
14-SPG-MCMACH-B-S10-300	1.7E+02	25	2	3	1.5	38	22.1
	1.7E+02	25	8	0	0.0	0	0.0
5-SPG-MCMACH-B-S13-300	2.6E+02	25	2	0	0.0	0	0.0
	2.6E+02	25	8	0	0.0	0	0.0
6-SPG-MCMACH-B-S17-300	2.4E+02	25.2	2	6	3.0	76	31.5
	2.4E+02	25.2	8	8	1.0	25	10.5
8-SPG-MCMACH-B-S04-3000	1.6E+03	25.8	2	20	10.0	258	16.1
	1.6E+03	25.8	8	3	0.4	10	0.6
15-SPG-MCMACH-B-S11-3000	1.7E+03	25	2	59	29.5	738	43.4
	1.7E+03	25	8	76	9.5	238	14.0
16-SPG-MCMACH-B-S12-3000	1.7E+03	25	2	0	0.0	0	0.0
	1.7E+03	25	8	0	0.0	0	0.0
7-SPG-MCMACH-B-S14-3000	2.6E+03	25.2	2	54	27.0	680	26.2
	2.6E+03	25.2	8	107	13.4	337	13.0
8-SPG-MCMACH-B-S18-3000	2.4E+03	24.2	2	84	42.0	1016	42.4
	2.4E+03	24.2	8	118	14.8	357	14.9

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Subway Car Filter Grill

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
9-SPG-SCGRIL-B-S01-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
1-SPG-SCGRIL-B-S05-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
9-SPG-SCGRIL-B-S13-0	0.0E+00	27	2	0	0.0	0	#DIV/0!
	0.0E+00	27	8	0	0.0	0	#DIV/0!
11-SPG-SCGRIL-B-S02-30	1.8E+01	24.5	2	0	0.0	0	0.0
	1.8E+01	24.5	8	0	0.0	0	0.0
2-SPG-SCGRIL-B-S06-30	3.3E+01	24	2	0	0.0	0	0.0
	3.3E+01	24	8	0	0.0	0	0.0
3-SPG-SCGRIL-B-S08-30	3.3E+01	24	2	0	0.0	0	0.0
	3.3E+01	24	8	0	0.0	0	0.0
12-SPG-SCGRIL-B-S14-30	3.4E+01	26.2	2	1	0.5	13	38.5
	3.4E+01	26.2	8	0	0.0	0	0.0
13-SPG-SCGRIL-B-S15-30	3.4E+01	24.2	2	0	0.0	0	0.0
	3.4E+01	24.2	8	0	0.0	0	0.0
13-SPG-SCGRIL-B-S03-300	1.8E+02	25	2	1	0.5	13	6.9
	1.8E+02	25	8	0	0.0	0	0.0
4-SPG-SCGRIL-B-S07-300	3.3E+02	25.4	2	9	4.5	114	34.6
	3.3E+02	25.4	8	18	2.3	57	17.3
5-SPG-SCGRIL-B-S09-300	3.3E+02	25	2	2	1.0	25	7.6
	3.3E+02	25	8	0	0.0	0	0.0
14-SPG-SCGRIL-B-S12-300	2.6E+02	24.4	2	8	4.0	98	37.5
	2.6E+02	24.4	8	21	2.6	64	24.6
14-SPG-SCGRIL-B-S16-300	3.4E+02	23.1	2	18	9.0	208	61.1
	3.4E+02	23.1	8	35	4.4	101	29.7
15-SPG-SCGRIL-B-S04-3000	1.8E+03	24.5	2	18	9.0	221	12.3
	1.8E+03	24.5	8	55	6.9	168	9.4
7-SPG-SCGRIL-B-S10-3000	3.3E+03	25.2	2	54	27.0	680	20.6
	3.3E+03	25.2	8	0	0.0	0	0.0
8-SPG-SCGRIL-B-S11-3000	3.3E+03	25	2	65	32.5	813	24.6
	3.3E+03	25	8	136	17.0	425	12.9
15-SPG-SCGRIL-B-S17-3000	3.4E+03	26	2	54	27.0	702	20.6
	3.4E+03	26	8	138	17.3	449	13.2
16-SPG-SCGRIL-B-S18-3000	3.4E+03	25	2	72	36.0	900	26.5
	3.4E+03	25	8	179	22.4	559	16.5

Use values highlighted in green for reporting.

Colony screened from this samples was negative for both real-time PCR targets

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Field Blank

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-SPNG-FLDBLK-A-S01-0	0.0E+00	22	2	0	0.0	0	#DIV/0!
	0.0E+00	22	8	0	0.0	0	#DIV/0!
1-SPG-FLDBLK-A-S11-0	0.0E+00	23.6	2	0	0.0	0	#DIV/0!
	0.0E+00	23.6	8	0	0.0	0	#DIV/0!
1-SPG-FLDBLK-A-S15-0	0.0E+00	26.2	2	0	0.0	0	#DIV/0!
	0.0E+00	26.2	8	0	0.0	0	#DIV/0!
3-SPNG-FLDBLK-A-S02-30	2.1E+01	22	2	1	0.5	11	52.4
	2.1E+01	22	8	6	0.8	17	78.6
4-SPG-FLDBLK-A-S05-30	1.7E+01	25.4	2	2	1.0	25	149.4
	1.7E+01	25.4	8	2	0.3	6	37.4
2-SPG-FLDBLK-A-S08-30	3.0E+01	24.4	2	1	0.5	12	40.7
	3.0E+01	24.4	8	2	0.3	6	20.3
9-SPG-FLDBLK-A-S12-30	2.4E+01	24.7	2	1	0.5	12	51.5
	2.4E+01	24.7	8	4	0.5	12	51.5
3-SPG-FLDBLK-A-S16-30	3.1E+01	24	2	0	0.0	0	0.0
	3.1E+01	24	8	7	0.9	21	67.7
5-SPNG-FLDBLK-A-S03-300	2.1E+02	22	2	8	4.0	88	41.9
	2.1E+02	22	8	17	2.1	47	22.3
6-SPG-FLDBLK-A-S06-300	3.3E+02	25	2	11	5.5	138	41.7
	3.3E+02	25	8	34	4.3	106	32.2
6-SPG-FLDBLK-A-S09-300	2.2E+02	25.8	2	9	4.5	116	52.8
	2.2E+02	25.8	8	38	4.8	123	55.7
11-SPG-FLDBLK-A-S13-300	2.4E+02	24.6	2	7	3.5	86	35.9
	2.4E+02	24.6	8	32	4.0	98	41.0
5-SPG-FLDBLK-A-S17-300	3.1E+02	24.8	2	10	5.0	124	40.0
	3.1E+02	24.8	8	33	4.1	102	33.0
7-SPNG-FLDBLK-A-S04-3000	2.1E+03	22	2	88	44.0	968	46.1
	2.1E+03	22	8	214	26.8	589	28.0
8-SPG-FLDBLK-A-S07-3000	3.1E+03	24.2	2	72	36.0	871	28.1
	3.1E+03	24.2	8	162	20.3	490	15.8
8-SPG-FLDBLK-A-S10-3000	2.4E+03	25.8	2	81	40.5	1045	43.5
	2.4E+03	25.8	8	185	23.1	597	24.9
14-SPG-FLDBLK-A-S14-3000	2.4E+03	24.2	2	76	38.0	920	38.3
	2.4E+03	24.2	8	206	25.8	623	26.0
7-SPG-FLDBLK-A-S18-3000	3.1E+03	25	2	60	30.0	750	24.2
	3.1E+03	25	8	202	25.3	631	20.4

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Sidewalk Concrete

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
2-SPNG-SWCON-A-S01-0	0.0E+00	22	2	1	0.5	11	#DIV/0!
	0.0E+00	22	8	1	0.1	3	#DIV/0!
9-SPG-SWCON-A-S05-0	0.0E+00	27	2	0	0.0	0	#DIV/0!
	0.0E+00	27	8	0	0.0	0	#DIV/0!
1-SPG-SWCON-A-S13-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
4-SPNG-SWCON-A-S02-30	2.1E+01	22	2	1	0.5	11	52.4
	2.1E+01	22	8	0	0.0	0	0.0
10-SPG-SWCON-A-S06-30	3.3E+01	24	2	1	0.5	12	36.4
	3.3E+01	24	8	0	0.0	0	0.0
11-SPG-SWCON-A-S07-30	3.3E+01	23.4	2	0	0.0	0	0.0
	3.3E+01	23.4	8	0	0.0	0	0.0
12-SPG-SWCON-A-S08-30	3.3E+01	24	2	0	0.0	0	0.0
	3.3E+01	24	8	0	0.0	0	0.0
2-SPG-SWCON-A-S14-30	3.3E+01	25.2	2	1	0.5	13	38.2
	3.3E+01	25.2	8	0	0.0	0	0.0
6-SPNG-SWCON-A-S03-300	2.1E+02	22	2	7	3.5	77	36.7
	2.1E+02	22	8	2	0.3	6	2.6
13-SPG-SWCON-A-S09-300	3.3E+02	24	2	2	1.0	24	7.3
	3.3E+02	24	8	0	0.0	0	0.0
14-SPG-SWCON-A-S10-300	3.3E+02	24	2	5	2.5	60	18.2
	3.3E+02	24	8	0	0.0	0	0.0
3-SPG-SWCON-A-S15-300	3.3E+02	24.6	2	8	4.0	98	29.8
	3.3E+02	24.6	8	0	0.0	0	0.0
4-SPG-SWCON-A-S16-300	3.3E+02	24.2	2	3	1.5	36	11.0
	3.3E+02	24.2	8	0	0.0	0	0.0
8-SPNG-SWCON-A-S04-3000	2.1E+03	22	2	54	27.0	594	28.3
	2.1E+03	22	8	0	0.0	0	0.0
15-SPG-SWCON-A-S11-3000	3.3E+03	24	2	26	13.0	312	9.5
	3.3E+03	24	8	0	0.0	0	0.0
16-SPG-SWCON-A-S12-3000	3.3E+03	25	2	15	7.5	188	5.7
	3.3E+03	25	8	0	0.0	0	0.0
7-SPG-SWCON-A-S17-3000	3.3E+03	25.6	2	43	21.5	550	16.7
	3.3E+03	25.6	8	102	12.8	326	9.9
8-SPG-SWCON-A-S18-3000	3.3E+03	24.2	2	33	16.5	399	12.1
	3.3E+03	24.2	8	120	15.0	363	11.0

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Electronic Display Panel, Above Ground

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
2-SPG-EDPAN(A)-A-S01-0	0.0E+00	26	2	0	0.0	0	#DIV/0!
	0.0E+00	26	8	0	0.0	0	#DIV/0!
1-SPG-EDPAN(A)-A-S05-0	0.0E+00	26	2	0	0.0	0	#DIV/0!
	0.0E+00	26	8	0	0.0	0	#DIV/0!
9-SPG-EDPAN(A)-A-S12-0	0.0E+00	24.4	2	0	0.0	0	#DIV/0!
	0.0E+00	24.4	8	0	0.0	0	#DIV/0!
4-SPG-EDPAN(A)-A-S02-30	1.8E+01	26.4	2	0	0.0	0	0.0
	1.8E+01	26.4	8	3	0.4	10	55.0
2-SPG-EDPAN(A)-A-S06-30	3.1E+01	25	2	0	0.0	0	0.0
	3.1E+01	25	8	0	0.0	0	0.0
3-SPG-EDPAN(A)-A-S07-30	3.1E+01	24.8	2	0	0.0	0	0.0
	3.1E+01	24.8	8	2	0.3	6	20.0
10-SPG-EDPAN(A)-A-S13-30	3.3E+01	24	2	2	1.0	24	72.7
	3.3E+01	24	8	0	0.0	0	0.0
11-SPG-EDPAN(A)-A-S14-30	3.3E+01	25	2	0	0.0	0	0.0
	3.3E+01	25	8	0	0.0	0	0.0
6-SPG-EDPAN(A)-A-S03-300	1.8E+02	23.2	2	6	3.0	70	38.7
	1.8E+02	23.2	8	0	0.0	0	0.0
4-SPG-EDPAN(A)-A-S08-300	3.1E+02	24.2	2	6	3.0	73	23.4
	3.1E+02	24.2	8	23	2.9	70	22.4
5-SPG-EDPAN(A)-A-S09-300	3.1E+02	24.2	2	8	4.0	97	31.2
	3.1E+02	24.2	8	11	1.4	33	10.7
12-SPG-EDPAN(A)-A-S15-300	3.3E+02	24.2	2	12	6.0	145	44.0
	3.3E+02	24.2	8	15	1.9	45	13.8
13-SPG-EDPAN(A)-A-S16-300	3.3E+02	24.4	2	9	4.5	110	33.3
	3.3E+02	24.4	8	25	3.1	76	23.1
8-SPG-EDPAN(A)-A-S04-3000	1.8E+03	25	2	44	22.0	550	30.6
	1.8E+03	25	8	105	13.1	328	18.2
6-SPG-EDPAN(A)-A-S10-3000	3.1E+03	24.4	2	36	18.0	439	14.2
	3.1E+03	24.4	8	104	13.0	317	10.2
7-SPG-EDPAN(A)-A-S11-3000	3.1E+03	24.2	2	39	19.5	472	15.2
	3.1E+03	24.2	8	116	14.5	351	11.3
15-SPG-EDPAN(A)-A-S17-3000	3.3E+03	25.4	2	87	43.5	1105	33.5
	3.3E+03	25.4	8	111	13.9	352	10.7
16-SPG-EDPAN(A)-A-S18-3000	3.3E+03	24	2	70	35.0	840	25.5
	3.3E+03	24	8	204	25.5	612	18.5

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Crosswalk Sign

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-SPG-CWSIGN-A-S01-0	0.0E+00	23.6	2	0	0.0	0	#DIV/0!
	0.0E+00	23.6	8	0	0.0	0	#DIV/0!
9-SPG-CWSIGN-A-S05-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
1-SPG-CWSIGN-A-S13-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
3-SPG-CWSIGN-A-S02-30	1.6E+01	24.4	2	0	0.0	0	0.0
	1.6E+01	24.4	8	0	0.0	0	0.0
10-SPG-CWSIGN-A-S06-30	3.1E+01	24	2	0	0.0	0	0.0
	3.1E+01	24	8	0	0.0	0	0.0
11-SPG-CWSIGN-A-S07-30	3.1E+01	24	2	0	0.0	0	0.0
	3.1E+01	24	8	0	0.0	0	0.0
12-SPG-CWSIGN-A-S08-30	3.1E+01	25	2	0	0.0	0	0.0
	3.1E+01	25	8	0	0.0	0	0.0
2-SPG-CWSIGN-A-S14-30	3.7E+01	24.2	2	2	1.0	24	65.4
	3.7E+01	24.2	8	0	0.0	0	0.0
5-SPG-CWSIGN-A-S03-300	1.6E+02	25	2	4	2.0	50	31.3
	1.6E+02	25	8	3	0.4	9	5.9
13-SPG-CWSIGN-A-S09-300	3.1E+02	24.8	2	4	2.0	50	16.0
	3.1E+02	24.8	8	4	0.5	12	4.0
14-SPG-CWSIGN-A-S10-300	3.1E+02	24.2	2	7	3.5	85	27.3
	3.1E+02	24.2	8	20	2.5	61	19.5
3-SPG-CWSIGN-A-S15-300	3.7E+02	24.2	2	3	1.5	36	9.8
	3.7E+02	24.2	8	11	1.4	33	9.0
4-SPG-CWSIGN-A-S16-300	3.7E+02	24.4	2	3	1.5	37	9.9
	3.7E+02	24.4	8	34	4.3	104	28.0
7-SPG-CWSIGN-A-S04-3000	1.6E+03	23.4	2	43	21.5	503	31.4
	1.6E+03	23.4	8	0	0.0	0	0.0
15-SPG-CWSIGN-A-S11-3000	3.1E+03	25	2	39	19.5	488	15.7
	3.1E+03	25	8	110	13.8	344	11.1
16-SPG-CWSIGN-A-S12-3000	3.1E+03	24.6	2	60	30.0	738	23.8
	3.1E+03	24.6	8	120	15.0	369	11.9
7-SPG-CWSIGN-A-S17-3000	3.7E+03	24	2	50	25.0	600	16.2
	3.7E+03	24	8	101	12.6	303	8.2
8-SPG-CWSIGN-A-S18-3000	3.7E+03	25.4	2	52	26.0	660	17.8
	3.7E+03	25.4	8	131	16.4	416	11.2

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Telephone Booth

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-SPG-TELEBO-A-S01-0	0.0E+00	25	2	7	3.5	88	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
1-SPG-TELEBO-A-S05-0	0.0E+00	24.4	2	0	0.0	0	#DIV/0!
	0.0E+00	24.4	8	0	0.0	0	#DIV/0!
9-SPG-TELEBO-A-S12-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
3-SPG-TELEBO-A-S02-30	3.1E+01	25	2	4	2.0	50	161.3
	3.1E+01	25	8	0	0.0	0	0.0
3-SPG-TELEBO-A-S06-30	3.0E+01	24.2	2	0	0.0	0	0.0
	3.0E+01	24.2	8	0	0.0	0	0.0
4-SPG-TELEBO-A-S07-30	3.0E+01	24.6	2	0	0.0	0	0.0
	3.0E+01	24.6	8	1	0.1	3	10.3
10-SPG-TELEBO-A-S13-30	3.7E+01	23.6	2	0	0.0	0	0.0
	3.7E+01	23.6	8	0	0.0	0	0.0
11-SPG-TELEBO-A-S14-30	3.7E+01	25	2	0	0.0	0	0.0
	3.7E+01	25	8	1	0.1	3	8.4
5-SPG-TELEBO-A-S03-300	3.1E+02	25	2	6	3.0	75	24.2
	3.1E+02	25	8	5	0.6	16	5.0
5-SPG-TELEBO-A-S08-300	3.0E+02	23.6	2	0	0.0	0	0.0
	3.0E+02	23.6	8	0	0.0	0	0.0
6-SPG-TELEBO-A-S09-300	3.0E+02	24.8	2	5	2.5	62	20.7
	3.0E+02	24.8	8	4	0.5	12	4.1
13-SPG-TELEBO-A-S15-300	3.7E+02	25	2	3	1.5	38	10.1
	3.7E+02	25	8	28	3.5	88	23.6
14-SPG-TELEBO-A-S16-300	3.7E+02	25.4	2	17	8.5	216	58.4
	3.7E+02	25.4	8	33	4.1	105	28.3
7-SPG-TELEBO-A-S04-3000	3.1E+03	25	2	55	27.5	688	22.2
	3.1E+03	25	8	35	4.4	109	3.5
7-SPG-TELEBO-A-S10-3000	3.0E+03	25.2	2	12	6.0	151	5.0
	3.0E+03	25.2	8	29	3.6	91	3.0
8-SPG-TELEBO-A-S11-3000	3.0E+03	25.6	2	42	21.0	538	17.9
	3.0E+03	25.6	8	83	10.4	266	8.9
15-SPG-TELEBO-A-S17-3000	3.7E+03	23.2	2	72	36.0	835	22.6
	3.7E+03	23.2	8	139	17.4	403	10.9
16-SPG-TELEBO-A-S18-3000	3.7E+03	24	2	69	34.5	828	22.4
	3.7E+03	24	8	124	15.5	372	10.1

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Street Grating

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
9-SPG-STGRAT-A-S01-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
9-SPG-STGRAT-A-S05-0	0.0E+00	24	2	0	0.0	0	#DIV/0!
	0.0E+00	24	8	0	0.0	0	#DIV/0!
2-SPG-STGRAT-A-S15-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
11-SPG-STGRAT-A-S02-30	1.9E+00	27	2	0	0.0	0	0.0
	1.9E+00	27	8	0	0.0	0	0.0
10-SPG-STGRAT-A-S06-30	3.0E+01	23	2	0	0.0	0	0.0
	3.0E+01	23	8	0	0.0	0	0.0
11-SPG-STGRAT-A-S07-30	3.0E+01	23.8	2	0	0.0	0	0.0
	3.0E+01	23.8	8	0	0.0	0	0.0
12-SPG-STGRAT-A-S08-30	3.0E+01	24.2	2	0	0.0	0	0.0
	3.0E+01	24.2	8	0	0.0	0	0.0
4-SPG-STGRAT-A-S16-30	3.1E+01	24.8	2	0	0.0	0	0.0
	3.1E+01	24.8	8	0	0.0	0	0.0
13-SPG-STGRAT-A-S03-300	1.9E+02	24.5	2	0	0.0	0	0.0
	1.9E+02	24.5	8	0	0.0	0	0.0
13-SPG-STGRAT-A-S09-300	3.0E+02	24.2	2	0	0.0	0	0.0
	3.0E+02	24.2	8	0	0.0	0	0.0
14-SPG-STGRAT-A-S10-300	3.0E+02	25.8	2	0	0.0	0	0.0
	3.0E+02	25.8	8	0	0.0	0	0.0
5-SPG-STGRAT-A-S13-300	3.3E+02	23.6	2	0	0.0	0	0.0
	3.3E+02	23.6	8	0	0.0	0	0.0
6-SPG-STGRAT-A-S17-300	3.1E+02	25.2	2	0	0.0	0	0.0
	3.1E+02	25.2	8	0	0.0	0	0.0
15-SPG-STGRAT-A-S04-3000	1.9E+03	26	2	0	0.0	0	0.0
	1.9E+03	26	8	0	0.0	0	0.0
15-SPG-STGRAT-A-S11-3000	3.0E+03	25	2	0	0.0	0	0.0
	3.0E+03	25	8	0	0.0	0	0.0
16-SPG-STGRAT-A-S12-3000	3.0E+03	24.2	2	0	0.0	0	0.0
	3.0E+03	24.2	8	0	0.0	0	0.0
6-SPG-STGRAT-A-S14-3000	3.3E+03	26	2	0	0.0	0	0.0
	3.3E+03	26	8	0	0.0	0	0.0
8-SPG-STGRAT-A-S18-3000	3.1E+03	24.2	2	0	0.0	0	0.0
	3.1E+03	24.2	8	0	0.0	0	0.0

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Crosswalk Painted

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
10-SPG-CWPNTD-A-S01-0	0.0E+00	25.5	2	0	0.0	0	#DIV/0!
	0.0E+00	25.5	8	0	0.0	0	#DIV/0!
10-SPG-CWPNTD-A-S05-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
1-SPG-CWPNTD-A-S09-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
12-SPG-CWPNTD-A-S02-30	1.9E+00	25	2	0	0.0	0	0.0
	1.9E+00	25	8	0	0.0	0	0.0
12-SPG-CWPNTD-A-S06-30	1.8E+01	26	2	0	0.0	0	0.0
	1.8E+01	26	8	0	0.0	0	0.0
2-SPG-CWPNTD-A-S10-30	2.2E+01	24.8	2	0	0.0	0	0.0
	2.2E+01	24.8	8	0	0.0	0	0.0
3-SPG-CWPNTD-A-S11-30	2.2E+01	25.2	2	0	0.0	0	0.0
	2.2E+01	25.2	8	0	0.0	0	0.0
10-SPG-CWPNTD-A-S16-30	2.4E+01	24.3	2	0	0.0	0	0.0
	2.4E+01	24.3	8	0	0.0	0	0.0
14-SPG-CWPNTD-A-S03-300	1.9E+02	25	2	1	0.5	13	6.6
	1.9E+02	25	8	0	0.0	0	0.0
14-SPG-CWPNTD-A-S07-300	1.8E+02	25	2	0	0.0	0	0.0
	1.8E+02	25	8	0	0.0	0	0.0
4-SPG-CWPNTD-A-S12-300	2.2E+02	24	2	0	0.0	0	0.0
	2.2E+02	24	8	0	0.0	0	0.0
5-SPG-CWPNTD-A-S13-300	2.2E+02	25	2	0	0.0	0	0.0
	2.2E+02	25	8	0	0.0	0	0.0
12-SPG-CWPNTD-A-S17-300	2.4E+02	24.4	2	3	1.5	37	15.3
	2.4E+02	24.4	8	0	0.0	0	0.0
16-SPG-CWPNTD-A-S04-3000	1.9E+03	24.5	2	0	0.0	0	0.0
	1.9E+03	24.5	8	0	0.0	0	0.0
16-SPG-CWPNTD-A-S08-3000	1.8E+03	25	2	17	8.5	213	11.8
	1.8E+03	25	8	0	0.0	0	0.0
7-SPG-CWPNTD-A-S14-3000	2.2E+03	23.2	2	0	0.0	0	0.0
	2.2E+03	23.2	8	0	0.0	0	0.0
8-SPG-CWPNTD-A-S15-3000	2.2E+03	25.2	2	18	9.0	227	10.3
	2.2E+03	25.2	8	85	10.6	268	12.2
15-SPG-CWPNTD-A-S18-3000	2.4E+03	25	2	34	17.0	425	17.7
	2.4E+03	25	8	118	14.8	369	15.4

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Sponge-Stick Granite Bench

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
10-SPG-GRNBEN-A-S01-0	0.0E+00	25	2	0	0.0	0	#DIV/0!
	0.0E+00	25	8	0	0.0	0	#DIV/0!
9-SPG-GRNBEN-A-S05-0	0.0E+00	25.3	2	0	0.0	0	#DIV/0!
	0.0E+00	25.3	8	0	0.0	0	#DIV/0!
9-SPG-GRNBEN-A-S15-0	0.0E+00	24.4	2	0	0.0	0	#DIV/0!
	0.0E+00	24.4	8	0	0.0	0	#DIV/0!
12-SPG-GRNBEN-A-S02-30	1.6E+01	23.2	2	0	0.0	0	0.0
	1.6E+01	23.2	8	0	0.0	0	0.0
10-SPG-GRNBEN-A-S06-30	2.2E+01	24.8	2	1	0.5	12	56.4
	2.2E+01	24.8	8	0	0.0	0	0.0
11-SPG-GRNBEN-A-S07-30	2.2E+01	23.4	2	0	0.0	0	0.0
	2.2E+01	23.4	8	0	0.0	0	0.0
12-SPG-GRNBEN-A-S08-30	2.2E+01	24.8	2	0	0.0	0	0.0
	2.2E+01	24.8	8	0	0.0	0	0.0
11-SPG-GRNBEN-A-S16-30	3.1E+01	23.6	2	1	0.5	12	38.1
	3.1E+01	23.6	8	1	0.1	3	9.5
14-SPG-GRBEN-A-S03-300	1.6E+02	25	2	1	0.5	13	7.8
	1.6E+02	25	8	13	1.6	41	25.4
13-SPG-GRNBEN-A-S09-300	2.2E+02	24.2	2	8	4.0	97	44.0
	2.2E+02	24.2	8	9	1.1	27	12.4
14-SPG-GRNBEN-A-S10-300	2.2E+02	24	2	4	2.0	48	21.8
	2.2E+02	24	8	21	2.6	63	28.6
5-SPG-GRNBEN-A-S13-300	3.7E+02	23.2	2	7	3.5	81	21.9
	3.7E+02	23.2	8	29	3.6	84	22.7
13-SPG-GRNBEN-A-S17-300	3.1E+02	24.4	2	5	2.5	61	19.7
	3.1E+02	24.4	8	4	0.5	12	3.9
16-SPG-GRBEN-A-S04-3000	1.6E+03	25	2	51	25.5	638	39.8
	1.6E+03	25	8	52	6.5	163	10.2
15-SPG-GRNBEN-A-S11-3000	2.2E+03	24	2	23	11.5	276	12.5
	2.2E+03	24	8	23	2.9	69	3.1
16-SPG-GRNBEN-A-S12-3000	2.2E+03	24.4	2	24	12.0	293	13.3
	2.2E+03	24.4	8	12	1.5	37	1.7
6-SPG-GRNBEN-A-S14-3000	3.7E+03	25.4	2	44	22.0	559	15.1
	3.7E+03	25.4	8	98	12.3	311	8.4
15-SPG-GRNBEN-A-S18-3000	3.1E+03	24	2	33	16.5	396	12.8
	3.1E+03	24	8	99	12.4	297	9.6

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

APPENDIX P: RV-PCR RESULTS FOR SPONGE-STICK SAMPLES USING CHROMOSOMAL AND pXO1 GENE TARGETS

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
1-SPG-FLDBLK-A-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-FLDBLK-A-S01-0 Tf		45	0			45	0		
2-SPG-SWCON-A-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-SWCON-A-S01-0 Tf		45	0			45	0		
3-SPG-FLDBLK-A-S02-30 T0	21	45	0	26.9	Pos.	45	0	27.3	Pos.
3-SPG-FLDBLK-A-S02-30 Tf		18.1	0.1			17.7	0		
4-SPG-SWCON-A-S02-30 T0	21	45	0	15.5	Pos.	45	0	17.5	Pos.
4-SPG-SWCON-A-S02-30 Tf		29.5	0.2			27.5	0.1		
5-SPG-FLDBLK-A-S03-300 T0	210	45	0	25.6	Pos.	45	0	25.8	Pos.
5-SPG-FLDBLK-A-S03-300 Tf		19.4	0.1			19.2	0		
6-SPG-SWCON-A-S03-300 T0	210	45	0	20.3	Pos.	45	0	20.9	Pos.
6-SPG-SWCON-A-S03-300 Tf		24.7	0			24.1	0		
7-SPG-FLDBLK-A-S04-3000 T0	2,100	45	0	25.7	Pos.	45	0	25.9	Pos.
7-SPG-FLDBLK-A-S04-3000 Tf		19.3	0.1			19.1	0		
8-SPG-SWCON-A-S04-3000 T0	2,100	45	0	21.3	Pos.	45	0	22.1	Pos.
8-SPG-SWCON-A-S04-3000 Tf		23.7	0			22.9	0		
9-SPG-GLSPAN-B-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-GLSPAN-B-S01-0 Tf		45	0			45	0		
10-SPG-WLTILE-B-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
10-SPG-WLTILE-B-S01-0 Tf		45	0			45	0		
11-SPG-GLSPAN-B-S02-30 T0	21	45	0	0	Neg.	45	0	0	Neg.
11-SPG-GLSPAN-B-S02-30 Tf		45	0			45	0		
12-SPG-WLTILE-B-S02-30 T0	21	45	0	0	Neg.	45	0	0	Neg.
12-SPG-WLTILE-B-S02-30 Tf		45	0			45	0		
13-SPG-GLSPAN-B-S03-300 T0	210	45	0	19.4	Pos.	45	0	20.2	Pos.
13-SPG-GLSPAN-B-S03-300 Tf		25.6	0.2			24.8	0.1		
14-SPG-WLTILE-B-S03-300 T0	210	45	0	21.8	Pos.	45	0	22.8	Pos.
14-SPG-WLTILE-B-S03-300 Tf		23.2	0.1			22.2	0		
15-SPG-GLSPAN-B-S04-3000 T0	2,100	45	0	26.5	Pos.	45	0	27.1	Pos.
15-SPG-GLSPAN-B-S04-3000 Tf		18.5	0.1			17.9	0		
16-SPG-WLTILE-B-S04-3000 T0	2,100	45	0	22.5	Pos.	45	0	23.7	Pos.
16-SPG-WLTILE-B-S04-3000 Tf		22.5	0.1			21.3	0		
1-SPG-TELEBO-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-TELEBO-0 Tf		45	0			45	0		
2-SPG-FLCON-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-FLCON-0 Tf		45	0			45	0		
3-SPG-TELEBO-30 T0	31	45	0	17.4	Pos.	45	0	17.8	Pos.
3-SPG-TELEBO-30 Tf		27.6	0.1			27.2	0		
4-SPG-FLCON-30 T0	31	45	0	14.8	Pos.	45	0	15.1	Pos.
4-SPG-FLCON-30 Tf		30.2	0			29.9	0		
5-SPG-TELEBO-300 T0	310	45	0	20.9	Pos.	45	0	20.9	Pos.
5-SPG-TELEBO-300 Tf		24.1	0.1			24.1	0		
6-SPG-FLCON-300 T0	310	45	0	18.7	Pos.	45	0	18.8	Pos.
6-SPG-FLCON-300 Tf		26.3	0.1			26.2	0.1		
7-SPG-TELEBO-3000 T0	3,100	45	0	22.9	Pos.	45	0	23.1	Pos.
7-SPG-TELEBO-3000 Tf		22.1	0.1			21.9	0		
8-SPG-FLCON-3000 T0	3,100	45	0	21.4	Pos.	45	0	21.4	Pos.
8-SPG-FLCON-3000 Tf		23.6	0			23.6	0		
9-SPG-FLLFIX-0 T0	0	45	0	0	Neg.	45	0	2.9	Neg.
9-SPG-FLLFIX-0 Tf		45	0			42.1	2.5		
10-SPG-FLTILE-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
10-SPG-FLTILE-0 TF		45	0			45	0		
11-SPG-FLLFIX-30 T0	31	45	0	16.2	Pos.	45	0	16	Pos.
11-SPG-FLLFIX-30 Tf		28.8	0			29	0.1		
12-SPG-FLTILE-30 T0	31	45	0	12.6	Pos.	45	0	12.5	Pos.
12-SPG-FLTILE-30 TF		32.4	0.1			32.5	0.1		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
13-SPG-FLLFIX-300 T0	310	45	0	18.8	Pos.	45	0	18.7	Pos.
13-SPG-FLLFIX-300 Tf		26.2	0.1			26.3	0.1		
14-SPG-FLTILE-300 T0	310	45	0	20.8	Pos.	45	0	21.2	Pos.
14-SPG-FLTILE-300 TF		24.2	0			23.8	0		
15-SPG-FLLFIX-3000 T0	3,100	45	0	23.2	Pos.	45	0	23.2	Pos.
15-SPG-FLLFIX-3000 Tf		21.8	0.1			21.8	0		
16-SPG-FLTILE-3000 T0	3,100	45	0	19.4	Pos.	44.5	0.8	19.4	Pos.
16-SPG-FLTILE-3000 TF		25.6	0			25.1	0		
1-SPG-CWSIGN-0 T0	0	45	0	1.9	Neg.	45	0	1.2	Neg.
1-SPG-CWSIGN-0 Tf		43.1	3.2			43.8	2.2		
2-SPG-MCMACH-0 T0	0	45	0	1.9	Neg.	45	0	1.2	Neg.
2-SPG-MCMACH-0 Tf		43.1	3.3			43.8	2.1		
3-SPG-CWSIGN-30 T0	16	45	0	18	Pos.	45	0	18	Pos.
3-SPG-CWSIGN-30 Tf		27	0			27	0		
4-SPG-MCMACH-30 T0	16	45	0	17.6	Pos.	45	0	17.7	Pos.
4-SPG-MCMACH-30 Tf		27.4	0			27.3	0.1		
5-SPG-CWSIGN-300 T0	160	45	0	22.9	Pos.	45	0	23	Pos.
5-SPG-CWSIGN-300 Tf		22.1	0			22	0.1		
6-SPG-MCMACH-300 T0	160	45	0	20.3	Pos.	44.9	0.1	20.7	Pos.
6-SPG-MCMACH-300 Tf		24.7	0			24.2	0		
7-SPG-CWSIGN-3000 T0	1,600	45	0	21.3	Pos.	45	0	21.4	Pos.
7-SPG-CWSIGN-3000 Tf		23.7	0.1			23.6	0.1		
8-SPG-MCMACH-3000 T0	1,600	45	0	21	Pos.	45	0	21.2	Pos.
8-SPG-MCMACH-3000 Tf		24	0			23.8	0		
9-SPG-OHSIGN-0 T0	0	45	0	0	Neg.	45	0	1.5	Neg.
9-SPG-OHSIGN-0 Tf		45	0			43.5	2.6		
10-SPG-GRNBEN-0 T0	0	45	0	1.5	Neg.	45	0	1.8	Neg.
10-SPG-GRNBEN-0 Tf		43.5	2.6			43.2	3.1		
11-SPG-OHSIGN-30 T0	16	45	0	16.9	Pos.	45	0	16.7	Pos.
11-SPG-OHSIGN-30 Tf		28.1	0.1			28.3	0		
12-SPG-GRNBEN-30 T0	16	45	0	17.3	Pos.	45	0	17.4	Pos.
12-SPG-GRNBEN-30 Tf		27.7	0.1			27.6	0		
13-SPG-OHSIGN-300 T0	160	45	0	21.4	Pos.	44.7	0.6	21.1	Pos.
13-SPG-OHSIGN-300 Tf		23.6	0.1			23.6	0		
14-SPG-GRBEN-300 T0	160	45	0	20.8	Pos.	45	0	21.1	Pos.
14-SPG-GRBEN-300 Tf		24.2	0			23.9	0		
15-SPG-OHSIGN-3000 T0	1,600	45	0	26.9	Pos.	43.7	1.4	25.7	Pos.
15-SPG-OHSIGN-3000 Tf		18.1	0			18	0.1		
16-SPG-GRBEN-3000 T0	1,600	45	0	24.1	Pos.	43.5	2.6	23.3	Pos.
16-SPG-GRBEN-3000 Tf		20.9	0			20.2	0		
1-SPG-STEPS-B-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-STEPS-B-S01-0 Tf		45	0			45	0		
2-SPG-EDPAN(B)-B-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-EDPAN(B)-B-0 Tf		45	0			45	0		
3-SPG-STEPS-30 T0	19	45	0	16.9	Pos.	44.3	1.2	16.4	Pos.
3-SPG-STEPS-30 Tf		28.1	0.1			27.9	0		
4-SPG-EDPAN(B)-30 T0	19	45	0	17.5	Pos.	45	0	17.5	Pos.
4-SPG-EDPAN(B)-30 Tf		27.5	0.1			27.5	0		
5-SPG-STEPS-300 T0	190	45	0	16.8	Pos.	45	0	17.1	Pos.
5-SPG-STEPS-300 Tf		28.2	0.1			27.9	0		
6-SPG-EDPAN(B)-300 T0	190	45	0	18.7	Pos.	45	0	18.8	Pos.
6-SPG-EDPAN(B)-300 Tf		26.3	0			26.2	0		
7-SPG-STEPS-3000 T0	1,900	45	0	21.7	Pos.	45	0	21.5	Pos.
7-SPG-STEPS-3000 Tf		23.3	0			23.5	0		
8-SPG-EDPAN(B)-3000 T0	1,900	45	0	26.1	Pos.	45	0	26.2	Pos.
8-SPG-EDPAN(B)-3000 Tf		18.9	0			18.8	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
9-SPG-STGRAT-0 T0	0	45	0	0	Neg.	44.7	0.5	-0.3	Neg.
9-SPG-STGRAT-0 Tf		45	0			45	0		
10-SPG-CWPNTD-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
10-SPG-CWPNTD-0 Tf		45	0			45	0		
11-SPG-STGRAT-30 T0	19	45	0	6.3	Neg.	43.2	3.2	6.5	Neg.
11-SPG-STGRAT-30 Tf		38.7	5.5			36.7	3		
12-SPG-CWPNTD-30 T0	19	45	0	6.6	Neg.	45	0	6.8	Neg.
12-SPG-CWPNTD-30 Tf		38.4	5.7			38.2	5.9		
13-SPG-STGRAT-300 T0	190	45	0	12	Pos.	45	0	12.4	Pos.
13-SPG-STGRAT-300 Tf		33	0.1			32.6	0		
14-SPG-CWPNTD-300 T0	190	45	0	13.5	Pos.	45	0	13.8	Pos.
14-SPG-CWPNTD-300 Tf		31.5	0.1			31.2	0.1		
15-SPG-STGRAT-3000 T0	1,900	45	0	19	Pos.	45	0	19.7	Pos.
15-SPG-STGRAT-3000 Tf		26	0			25.3	0		
16-SPG-CWPNTD-3000 T0	1,900	45	0	16.6	Pos.	45	0	16.8	Pos.
16-SPG-CWPNTD-3000 Tf		28.4	0.1			28.2	0		
1-SPG-GLSWIN-B-S01-0 T0	0	45	0	0	Neg.	45	0	0.2	Neg.
1-SPG-GLSWIN-B-S01-0 Tf		45	0			44.8	0.3		
2-SPG-EDPAN(A)-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-EDPAN(A)-S01-0 Tf		45	0			45	0		
3-SPG-GLSWIN-S02-30 T0	18	45	0	20.4	Pos.	45	0	20.1	Pos.
3-SPG-GLSWIN-S02-30 Tf		24.6	0.1			24.9	0		
4-SPG-EDPAN(A)-30 T0	18	45	0	16.6	Pos.	45	0	16.8	Pos.
4-SPG-EDPAN(A)-30 Tf		28.4	0			28.2	0		
5-SPG-GLSWIN-300 T0	180	45	0	26.8	Pos.	45	0	27.1	Pos.
5-SPG-GLSWIN-300 Tf		18.2	0			17.9	0		
6-SPG-EDPAN(A)-300 T0	180	45	0	23.1	Pos.	45	0	23.1	Pos.
6-SPG-EDPAN(A)-300 Tf		21.9	0			21.9	0		
7-SPG-GLSWIN-3000 T0	1,800	45	0	26	Pos.	45	0	26	Pos.
7-SPG-GLSWIN-3000 Tf		19	0			19	0		
8-SPG-EDPAN(A)-3000 T0	1,800	45	0	26.7	Pos.	44.7	0.5	26.7	Pos.
8-SPG-EDPAN(A)-3000 Tf		18.3	0			17.9	0		
9-SPG-SCGRIL-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-SCGRIL-0 Tf		45	0			45	0		
10-SPG-CWPNTD-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
10-SPG-CWPNTD-0 Tf		45	0			45	0		
11-SPG-SCGRIL-30 T0	18	45	0	14.3	Pos.	44.5	0.8	14	Pos.
11-SPG-SCGRIL-30 Tf		30.7	0.2			30.5	0		
12-SPG-CWPNTD-30 T0	18	45	0	2.3	Neg.	45	0	6.8	Neg.
12-SPG-CWPNTD-30 Tf		42.7	3.9			38.2	1		
13-SPG-SCGRIL-300 T0	180	45	0	8.6	Neg.	45	0	9.4	Neg.
13-SPG-SCGRIL-300 Tf		36.4	1.1			35.6	0.2		
14-SPG-CWPNTD-300 T0	180	45	0	14.8	Pos.	45	0	15.1	Pos.
14-SPG-CWPNTD-300 Tf		30.2	0.1			29.9	0		
15-SPG-SCGRIL-3000 T0	1,800	45	0	20.6	Pos.	45	0	21.1	Pos.
15-SPG-SCGRIL-3000 Tf		24.4	0			23.9	0		
16-SPG-CWPNTD-3000 T0	1,800	45	0	16.3	Pos.	45	0	16.7	Pos.
16-SPG-CWPNTD-3000 Tf		28.7	0			28.3	0		
1-SPG-FLTILE-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-FLTILE-B-S05-0 Tf		45	0			45	0		
2-SPG-FLCON-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-FLCON-B-S05-0 Tf		45	0			45	0		
3-SPG-FLTILE-B-S06-30 T0	23	45	0	10.2	Pos.	45	0	9.9	Pos.
3-SPG-FLTILE-B-S06-30 Tf		34.8	0.1			35.1	0.2		
4-SPG-FLCON-B-S06-30 T0	23	45	0	14	Pos.	45	0	14.1	Pos.
4-SPG-FLCON-B-S06-30 Tf		31	0			30.9	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
5-SPG-FLTILE-B-S07-300 T0	230	45	0	19.7	Pos.	45	0	19.7	Pos.
5-SPG-FLTILE-B-S07-300 Tf		25.3	0			25.3	0		
6-SPG-FLCON-S07-300 T0	230	45	0	15.4	Pos.	43.3	3	14.1	Pos.
6-SPG-FLCON-S07-300 Tf		29.6	0.1			29.2	0		
7-SPG-FLTILE-S08-3000 T0	2,300	45	0	22.4	Pos.	45	0	22.4	Pos.
7-SPG-FLTILE-S08-3000 Tf		22.6	0			22.6	0.1		
8-SPG-FLCON-S08-3000 T0	2,300	45	0	22	Pos.	45	0	22.2	Pos.
8-SPG-FLCON-S08-3000 Tf		23	0			22.8	0		
9-SPG-STEPS-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-STEPS-B-S05-0 Tf		45	0			45	0		
10-SPG-WLTILE-S05-0 T0	0	45	0	0	Neg.	45	0	0.5	Neg.
10-SPG-WLTILE-S05-0 Tf		45	0			44.5	0.9		
11-SPG-STEPS-S06-30 T0	23	45	0	17.2	Pos.	45	0	17.3	Pos.
11-SPG-STEPS-S06-30 Tf		27.8	0.1			27.7	0		
12-SPG-WLTILE-S06-30 T0	23	45	0	21.4	Pos.	45	0	21.5	Pos.
12-SPG-WLTILE-S06-30 Tf		23.6	0			23.5	0		
13-SPG-STEPS-S07-300 T0	230	45	0	23.2	Pos.	45	0	23.6	Pos.
13-SPG-STEPS-S07-300 Tf		21.8	0.1			21.4	0.1		
14-SPG-WLTILE-07-300 T0	230	45	0	25.8	Pos.	45	0	26.2	Pos.
14-SPG-WLTILE-07-300 Tf		19.2	0			18.8	0		
15-SPG-STEPS-08-3000 T0	2,300	45	0	23.6	Pos.	45	0	23.4	Pos.
15-SPG-STEPS-08-3000 Tf		21.4	0			21.6	0		
16-SPG-WLTILE-3000 T0	2,300	45	0	22.8	Pos.	45	0	23.1	Pos.
16-SPG-WLTILE-3000 Tf		22.2	0			21.9	0		
1-SPG-GLSWIN-B-S05-0 T0	0	45	0	3.1	Neg.	45	0	1.5	Neg.
1-SPG-GLSWIN-B-S05-0 Tf		41.9	3.4			43.5	1.3		
2-SPG-GLSWIN-B-S06-30 T0	34	45	0	23.6	Pos.	45	0	23.2	Pos.
2-SPG-GLSWIN-B-S06-30 Tf		21.4	0.1			21.8	0.1		
3-SPG-GLSWIN-B-S07-30 T0	34	45	0	18.2	Pos.	45	0	18.2	Pos.
3-SPG-GLSWIN-B-S07-30 Tf		26.8	0.1			26.8	0		
4-SPG-GLSWIN-B-S08-30 T0	34	45	0	18	Pos.	45	0	18.1	Pos.
4-SPG-GLSWIN-B-S08-30 Tf		27	0			26.9	0		
5-SPG-GLSWIN-B-S09-300 T0	340	45	0	24.2	Pos.	45	0	24.3	Pos.
5-SPG-GLSWIN-B-S09-300 Tf		20.8	0.1			20.7	0.1		
6-SPG-GLSWIN-B-S10-300 T0	340	45	0	17	Pos.	45	0	17	Pos.
6-SPG-GLSWIN-B-S10-300 Tf		28	0.1			28	0.1		
7-SPG-GLSWIN-B-S11-3000 T0	3,400	45	0	25.3	Pos.	45	0	25.4	Pos.
7-SPG-GLSWIN-B-S11-3000 Tf		19.7	0.1			19.6	0.1		
8-SPG-GLSWIN-B-S12-3000 T0	3,400	45	0	27.6	Pos.	45	0	27.9	Pos.
8-SPG-GLSWIN-B-S12-3000 Tf		17.4	0			17.1	0		
9-SPG-EDPAN(B)-S05-0 T0	0	45	0	0	Neg.	45	0	1.3	Neg.
9-SPG-EDPAN(B)-S05-0 Tf		45	0			43.7	2.1		
10-SPG-EDPAN(B)-S06-30 T0	34	45	0	19.8	Pos.	45	0	19.6	Pos.
10-SPG-EDPAN(B)-S06-30 Tf		25.2	0			25.4	0		
11-SPG-EDPAN(B)-S07-30 T0	34	45	0	23.7	Pos.	45	0	23.9	Pos.
11-SPG-EDPAN(B)-S07-30 Tf		21.3	0			21.1	0		
12-SPG-EDPAN(B)-S08-30 T0	34	45	0	21	Pos.	45	0	21.1	Pos.
12-SPG-EDPAN(B)-S08-30 Tf		24	0			23.9	0		
13-SPG-EDPAN(B)-S09-300 T0	340	45	0	20	Pos.	44.5	0.8	19.6	Pos.
13-SPG-EDPAN(B)-S09-300 Tf		25	0			25	0		
14-SPG-EDPAN(B)-S10-300 T0	340	45	0	22.3	Pos.	45	0	22.4	Pos.
14-SPG-EDPAN(B)-S10-300 Tf		22.7	0			22.6	0		
15-SPG-EDPAN(B)-S11-3000 T0	3,400	45	0	19.9	Pos.	45	0	20	Pos.
15-SPG-EDPAN(B)-S11-3000 Tf		25.1	0			25	0		
16-SPG-EDPAN(B)-S12-3000 T0	3,400	45	0	24.7	Pos.	45	0	25.1	Pos.
16-SPG-EDPAN(B)-S12-3000 Tf		20.3	0.1			19.9	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
1-SPG-GLSPAN-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-GLSPAN-B-S05-0 Tf		45	0			45	0		
2-SPG-GLSPAN-B-S06-30 T0	48	45	0	20	Pos.	45	0	19.9	Pos.
2-SPG-GLSPAN-B-S06-30 Tf		25	0.1			25.1	0		
3-SPG-GLSPAN-B-S07-30 T0	48	45	0	17.6	Pos.	45	0	17.5	Pos.
3-SPG-GLSPAN-B-S07-30 Tf		27.4	0			27.5	0		
4-SPG-GLSPAN-B-S08-30 T0	48	45	0	23.4	Pos.	45	0	23.3	Pos.
4-SPG-GLSPAN-B-S08-30 Tf		21.6	0.1			21.7	0		
5-SPG-GLSPAN-B-S09-300 T0	480	45	0	25.2	Pos.	45	0	25.1	Pos.
5-SPG-GLSPAN-B-S09-300 Tf		19.8	0.1			19.9	0		
6-SPG-GLSPAN-B-S10-300 T0	480	45	0	22.2	Pos.	45	0	22.1	Pos.
6-SPG-GLSPAN-B-S10-300 Tf		22.8	0			22.9	0		
7-SPG-GLSPAN-B-S11-3000 T0	4,800	40.5	3.9	20.2	Pos.	44.5	0.9	24	Pos.
7-SPG-GLSPAN-B-S11-3000 Tf		20.3	0			20.5	0		
8-SPG-GLSPAN-B-S12-3000 T0	4,800	45	0	24.9	Pos.	45	0	24.8	Pos.
8-SPG-GLSPAN-B-S12-3000 Tf		20.1	0			20.2	0		
9-SPG-FLLFIX-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-FLLFIX-B-S05-0 Tf		45	0			45	0		
10-SPG-FLLFIX-B-S06-30 T0	48	45	0	15.1	Pos.	45	0	15.2	Pos.
10-SPG-FLLFIX-B-S06-30 Tf		29.9	0.1			29.8	0		
11-SPG-FLLFIX-B-S07-30 T0	48	45	0	25.8	Pos.	45	0	25.6	Pos.
11-SPG-FLLFIX-B-S07-30 Tf		19.2	0			19.4	0		
12-SPG-FLLFIX-B-S08-30 T0	48	45	0	16.1	Pos.	45	0	16.2	Pos.
12-SPG-FLLFIX-B-S08-30 Tf		28.9	0.1			28.8	0		
13-SPG-FLLFIX-B-S09-300 T0	480	42.7	4	18.9	Pos.	42.6	3.2	19	Pos.
13-SPG-FLLFIX-B-S09-300 Tf		23.7	0			23.7	0		
14-SPG-FLLFIX-B-S10-300 T0	480	42.9	3.6	16.4	Pos.	40.5	2.6	14.1	Pos.
14-SPG-FLLFIX-B-S10-300 Tf		26.5	0.1			26.4	0		
15-SPG-FLLFIX-B-S11-3000 T0	4,800	45	0	23.9	Pos.	44.6	0.7	23.5	Pos.
15-SPG-FLLFIX-B-S11-3000 Tf		21.1	0.1			21.1	0		
16-SPG-FLLFIX-B-S12-3000 T0	4,800	45	0	24	Pos.	45	0	24.5	Pos.
16-SPG-FLLFIX-B-S12-3000 Tf		21	0			20.5	0		
1-SPG-OHSIGN-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-OHSIGN-B-S05-0 Tf		45	0			45	0		
2-SPG-OHSIGN-B-S06-30 T0	17	43	3.4	18.5	Pos.	45	0	20.4	Pos.
2-SPG-OHSIGN-B-S06-30 Tf		24.5	0			24.6	0		
3-SPG-OHSIGN-B-S07-30 T0	17	45	0	12.8	Pos.	45	0	12.9	Pos.
3-SPG-OHSIGN-B-S07-30 Tf		32.2	0.2			32.1	0.1		
4-SPG-FLDBLK-A-S05-30 T0	17	45	0	26.1	Pos.	42.1	2.6	23.1	Pos.
4-SPG-FLDBLK-A-S05-30 Tf		18.9	0			19	0		
5-SPG-OHSIGN-B-S08-300 T0	170	45	0	21.5	Pos.	45	0	21.7	Pos.
5-SPG-OHSIGN-B-S08-300 Tf		23.5	0.1			23.3	0		
6-SPG-OHSIGN-B-S09-300 T0	170	45	0	25.5	Pos.	45	0	25.5	Pos.
6-SPG-OHSIGN-B-S09-300 Tf		19.5	0.2			19.5	0.2		
7-SPG-OHSIGN-B-S10-3000 T0	1,700	45	0	26.2	Pos.	45	0	26.1	Pos.
7-SPG-OHSIGN-B-S10-3000 Tf		18.8	0			18.9	0		
8-SPG-OHSIGN-B-S11-3000 T0	1,700	45	0	22.7	Pos.	45	0	22.7	Pos.
8-SPG-OHSIGN-B-S11-3000 Tf		22.3	0.7			22.3	0.7		
9-SPG-MCMACH-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-MCMACH-B-S05-0 Tf		45	0			45	0		
10-SPG-MCMACH-B-S06-30 T0	17	45	0	12.1	Pos.	45	0	12	Pos.
10-SPG-MCMACH-B-S06-30 Tf		32.9	0.2			33	0.2		
11-SPG-MCMACH-B-S07-30 T0	17	45	0	9.9	Pos.	45	0	10	Pos.
11-SPG-MCMACH-B-S07-30 Tf		35.1	0.3			35	0.1		
12-SPG-MCMACH-B-S08-30 T0	17	45	0	15.6	Pos.	45	0	15.7	Pos.
12-SPG-MCMACH-B-S08-30 Tf		29.4	0			29.3	0.1		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
13-SPG-MCMACH-B-S09-300 T0	170	45	0	18.2	Pos.	45	0	18.2	Pos.
13-SPG-MCMACH-B-S09-300 Tf		26.8	0			26.8	0		
14-SPG-MCMACH-B-S10-300 T0	170	45	0	18	Pos.	45	0	18.5	Pos.
14-SPG-MCMACH-B-S10-300 Tf		27	0			26.5	0		
15-SPG-MCMACH-B-S11-3000 T0	1,700	45	0	19.2	Pos.	45	0	19.5	Pos.
15-SPG-MCMACH-B-S11-3000 Tf		25.8	0			25.5	0.1		
16-SPG-MCMACH-B-S12-3000 T0	1,700	45	0	20.2	Pos.	45	0	20.5	Pos.
16-SPG-MCMACH-B-S12-3000 Tf		24.8	0			24.5	0		
1-SPG-SCGRIL-B-S05-0 T0	0	45	0	0	Neg.	45	0	1.5	Neg.
1-SPG-SCGRIL-B-S05-0 Tf		45	0			43.5	2.6		
2-SPG-SCGRIL-B-S06-30 T0	33	45	0	17.8	Pos.	45	0	17.9	Pos.
2-SPG-SCGRIL-B-S06-30 Tf		27.2	0.1			27.1	0.1		
3-SPG-SCGRIL-B-S08-30 T0	33	45	0	14.9	Pos.	45	0	14.9	Pos.
3-SPG-SCGRIL-B-S08-30 Tf		30.1	0.1			30.1	0.1		
4-SPG-SCGRIL-B-S07-300 T0	330	45	0	21.7	Pos.	45	0	21.9	Pos.
4-SPG-SCGRIL-B-S07-300 Tf		23.3	0			23.1	0		
5-SPG-SCGRIL-B-S09-300 T0	330	45	0	15.6	Pos.	45	0	15.8	Pos.
5-SPG-SCGRIL-B-S09-300 Tf		29.4	0			29.2	0		
6-SPG-FLDBLK-S06-300 T0	330	45	0	26	Pos.	45	0	26.2	Pos.
6-SPG-FLDBLK-S06-300 Tf		19	0			18.8	0		
7-SPG-SCGRIL-B-S10-3000 T0	3,300	45	0	21	Pos.	45	0	20.4	Pos.
7-SPG-SCGRIL-B-S10-3000 Tf		24	0			24.6	0		
8-SPG-SCGRIL-B-S11-3000 T0	3,300	45	0	19.7	Pos.	45	0	19.8	Pos.
8-SPG-SCGRIL-B-S11-3000 Tf		25.3	0.1			25.2	0		
9-SPG-SWCON-A-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-SWCON-A-S05-0 Tf		45	0			45	0		
10-SPG-SWCON-A-S06-30 T0	33	45	0	14.8	Pos.	45	0	14.8	Pos.
10-SPG-SWCON-A-S06-30 Tf		30.2	0.1			30.2	0.1		
11-SPG-SWCON-A-S07-30 T0	33	45	0	13.3	Pos.	45	0	13.1	Pos.
11-SPG-SWCON-A-S07-30 Tf		31.7	0			31.9	0.1		
12-SPG-SWCON-A-S08-30 T0	33	45	0	17.4	Pos.	45	0	17.6	Pos.
12-SPG-SWCON-A-S08-30 Tf		27.6	0			27.4	0		
13-SPG-SWCON-A-S09-300 T0	330	45	0	13.6	Pos.	45	0	14.1	Pos.
13-SPG-SWCON-A-S09-300 Tf		31.4	0.1			30.9	0		
14-SPG-SWCON-A-S10-300 T0	330	45	0	18.3	Pos.	45	0	19.1	Pos.
14-SPG-SWCON-A-S10-300 Tf		26.7	0			25.9	0		
15-SPG-SWCON-A-S11-3000 T0	3,300	45	0	21.6	Pos.	45	0	21.9	Pos.
15-SPG-SWCON-A-S11-3000 Tf		23.4	0			23.1	0		
16-SPG-SWCON-A-S12-3000 T0	3,300	45	0	23.5	Pos.	45	0	24.1	Pos.
16-SPG-SWCON-A-S12-3000 Tf		21.5	0			20.9	0		
1-SPG-EDPAN(A)-A-S05-0 T0	0	45	0	0	Neg.	45	0	3	Neg.
1-SPG-EDPAN(A)-A-S05-0 Tf		45	0			42	2.6		
2-SPG-EDPAN(A)-A-S06-30 T0	31	45	0	17.5	Pos.	45	0	17.2	Pos.
2-SPG-EDPAN(A)-A-S06-30 Tf		27.5	0.1			27.8	0.1		
3-SPG-EDPAN(A)-A-S07-30 T0	31	45	0	24.1	Pos.	45	0	24.1	Pos.
3-SPG-EDPAN(A)-A-S07-30 Tf		20.9	0.1			20.9	0		
4-SPG-EDPAN(A)-A-S08-300 T0	310	45	0	21.9	Pos.	45	0	21.7	Pos.
4-SPG-EDPAN(A)-A-S08-300 Tf		23.1	0			23.3	0.1		
5-SPG-EDPAN(A)-A-S09-300 T0	310	45	0	20.1	Pos.	45	0	19.7	Pos.
5-SPG-EDPAN(A)-A-S09-300 Tf		24.9	0			25.3	0.1		
6-SPG-EDPAN(A)-A-S10-3000 T0	3,100	45	0	23.4	Pos.	45	0	23.3	Pos.
6-SPG-EDPAN(A)-A-S10-3000 Tf		21.6	0			21.7	0		
7-SPG-EDPAN(A)-A-S11-3000 T0	3,100	45	0	24.2	Pos.	45	0	24.1	Pos.
7-SPG-EDPAN(A)-A-S11-3000 Tf		20.8	0.1			20.9	0		
8-SPG-FLDBLK-A-S07-3000 T0	3,100	45	0	25.4	Pos.	45	0	25.4	Pos.
8-SPG-FLDBLK-A-S07-3000 Tf		19.6	0			19.6	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
9-SPG-CWSIGN-A-05-0 T0	0	45	0	0	Neg.	45	0	0.8	Neg.
9-SPG-CWSIGN-A-05-0 Tf		45	0			44.2	1.3		
10-SPG-CWSIGN-A-06-30 T0	31	45	0	18.8	Pos.	45	0	19	Pos.
10-SPG-CWSIGN-A-06-30 Tf		26.2	0.1			26	0.1		
11-SPG-CWSIGN-A-07-30 T0	31	45	0	12.8	Pos.	45	0	13	Pos.
11-SPG-CWSIGN-A-07-30 Tf		32.2	0.1			32	0		
12-SPG-CWSIGN-A-08-30 T0	31	45	0	16.7	Pos.	45	0	17	Pos.
12-SPG-CWSIGN-A-08-30 Tf		28.3	0			28	0		
13-SPG-CWSIGN-A-09-300 T0	310	45	0	24.9	Pos.	45	0	24.6	Pos.
13-SPG-CWSIGN-A-09-300 Tf		20.1	0.1			20.4	0		
14-SPG-CWSIGN-A-10-300 T0	310	45	0	16.3	Pos.	45	0	16.3	Pos.
14-SPG-CWSIGN-A-10-300 Tf		28.7	0			28.7	0.1		
15-SPG-CWSIGN-A-11-3000 T0	3,100	45	0	24.5	Pos.	45	0	24.6	Pos.
15-SPG-CWSIGN-A-11-3000 Tf		20.5	0			20.4	0		
16-SPG-CWSIGN-A-12-3000 T0	3,100	45	0	26.1	Pos.	45	0	26.2	Pos.
16-SPG-CWSIGN-A-12-3000 Tf		18.9	0			18.8	0		
1-SPG-TELEBO-A-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-TELEBO-A-S05-0 Tf		45	0			45	0		
2-SPG-FLDBLK-A-S08-30 T0	30	45	0	27.4	Pos.	45	0	27.1	Pos.
2-SPG-FLDBLK-A-S08-30 Tf		17.6	0			17.9	0		
3-SPG-TELEBO-A-S06-30 T0	30	45	0	18.2	Pos.	45	0	18.1	Pos.
3-SPG-TELEBO-A-S06-30 Tf		26.8	0.1			26.9	0		
4-SPG-TELEBO-A-S07-30 T0	30	45	0	19.8	Pos.	45	0	19.9	Pos.
4-SPG-TELEBO-A-S07-30 Tf		25.2	0			25.1	0		
5-SPG-TELEBO-A-S08-300 T0	300	45	0	15.3	Pos.	43.6	2.5	14.2	Pos.
5-SPG-TELEBO-A-S08-300 Tf		29.7	0.1			29.4	0		
6-SPG-TELEBO-A-S09-300 T0	300	45	0	17.9	Pos.	45	0	17.8	Pos.
6-SPG-TELEBO-A-S09-300 Tf		27.1	0			27.2	0		
7-SPG-TELEBO-A-S10-3000 T0	3,000	45	0	23	Pos.	45	0	22.9	Pos.
7-SPG-TELEBO-A-S10-3000 Tf		22	0			22.1	0		
8-SPG-TELEBO-A-S11-3000 T0	3,000	42.1	5	21.9	Pos.	42.2	4.9	22.2	Pos.
8-SPG-TELEBO-A-S11-3000 Tf		20.2	0			19.9	0		
9-SPG-STGRAT-A-S05-0 T0	0	45	0	7.5	Neg.	45	0	7	Neg.
9-SPG-STGRAT-A-S05-0 Tf		37.5	0.9			38	0.7		
10-SPG-STGRAT-A-S06-30 T0	30	45	0	10.1	Pos.	45	0	10.3	Pos.
10-SPG-STGRAT-A-S06-30 Tf		34.9	0.3			34.7	0.1		
11-SPG-STGRAT-A-S07-30 T0	30	41.3	6.5	7.8	Neg.	45	0	12.1	Neg.*
11-SPG-STGRAT-A-S07-30 Tf		33.5	0.3			32.9	0.1		
12-SPG-STGRAT-A-S08-30 T0	30	42.7	4.1	7.9	Neg.	42.6	4.1	8.7	Neg.
12-SPG-STGRAT-A-S08-30 Tf		34.7	0.1			33.9	0.1		
13-SPG-STGRAT-A-S09-300 T0	300	45	0	14.7	Pos.	45	0	15.1	Pos.
13-SPG-STGRAT-A-S09-300 Tf		30.3	0.2			29.9	0.1		
14-SPG-STGRAT-A-S10-300 T0	300	45	0	12.5	Pos.	45	0	13	Pos.
14-SPG-STGRAT-A-S10-300 Tf		32.5	0			32	0		
15-SPG-STGRAT-A-S11-3000 T0	3,000	45	0	18.3	Pos.	45	0	18.7	Pos.
15-SPG-STGRAT-A-S11-3000 Tf		26.7	0			26.3	0.1		
16-SPG-STGRAT-A-S12-3000 T0	3,000	45	0	16.8	Pos.	45	0	17.6	Pos.
16-SPG-STGRAT-A-S12-3000 Tf		28.2	0.1			27.4	0		
1-SPG-CWPNTD-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-CWPNTD-0 Tf		45	0			45	0		
2-SPG-CWPNTD-30 T0	22	45	0	20.3	Pos.	42.9	2.1	18	Pos.
2-SPG-CWPNTD-30 Tf		24.7	0.1			24.9	0		
3-SPG-CWPNTD-30 T0	22	45	0	12.6	Pos.	45	0	12.5	Pos.
3-SPG-CWPNTD-30 Tf		32.4	0.2			32.5	0		
4-SPG-CWPNTD-300 T0	220	45	0	23.4	Pos.	45	0	23.6	Pos.
4-SPG-CWPNTD-300 Tf		21.6	0			21.4	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
5-SPG-CWPNTD-300 T0	220	45	0	21.3	Pos.	45	0	21.4	Pos.
5-SPG-CWPNTD-300 Tf		23.7	0.1			23.6	0		
6-SPG-FLDBLK-300 T0	220	45	0	25.3	Pos.	45	0	25.2	Pos.
6-SPG-FLDBLK-300 Tf		19.7	0			19.8	0		
7-SPG-CWPNTD-3000 T0	2,200	45	0	25.5	Pos.	45	0	25.3	Pos.
7-SPG-CWPNTD-3000 Tf		19.5	0			19.7	0		
8-SPG-CWPNTD-3000 T0	2,200	43.2	3.2	21.1	Pos.	44.4	1	22.3	Pos.
8-SPG-CWPNTD-3000 Tf		22	0			22.1	0		
9-SPG-GRNBEN-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-GRNBEN-0 Tf		45	0			45	0		
10-SPG-GRNBEN-30 T0	22	45	0	11.7	Pos.	45	0	11.3	Pos.
10-SPG-GRNBEN-30 Tf		33.3	0.2			33.7	0.2		
11-SPG-GRNBEN-30 T0	22	45	0	9.8	Neg.*	43.5	2.6	7.9	Neg.
11-SPG-GRNBEN-30 Tf		35.2	0.3			35.6	0.1		
12-SPG-GRNBEN-30 T0	22	45	0	15.4	Pos.	45	0	15.3	Pos.
12-SPG-GRNBEN-30 Tf		29.6	0			29.7	0		
13-SPG-GRNBEN-300 T0	220	45	0	18.1	Pos.	45	0	17.9	Pos.
13-SPG-GRNBEN-300 Tf		26.9	0.1			27.1	0		
14-SPG-GRNBEN-300 T0	220	45	0	17.8	Pos.	45	0	18.1	Pos.
14-SPG-GRNBEN-300 Tf		27.2	0.1			26.9	0		
15-SPG-GRNBEN-3000 T0	2,200	45	0	19.2	Pos.	45	0	19.2	Pos.
15-SPG-GRNBEN-3000 Tf		25.8	0.1			25.8	0		
16-SPG-GRNBEN-3000 T0	2,200	45	0	19.7	Pos.	45	0	19.9	Pos.
16-SPG-GRNBEN-3000 Tf		25.3	0			25.1	0		
1-SPG-FLTILE-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-FLTILE-0 Tf		45	0			45	0		
2-SPG-FLTILE-30 T0	24	45	0	11.7	Pos.	45	0	11.3	Pos.
2-SPG-FLTILE-30 Tf		33.3	0.3			33.7	0.2		
3-SPG-FLTILE-30 T0	24	45	0	13.5	Pos.	45	0	13.3	Pos.
3-SPG-FLTILE-30 Tf		31.5	0			31.7	0		
4-SPG-FLTILE-300 T0	240	45	0	20.3	Pos.	45	0	20.3	Pos.
4-SPG-FLTILE-300 Tf		24.7	0			24.7	0		
5-SPG-FLTILE-300 T0	240	45	0	14	Pos.	45	0	13.3	Pos.
5-SPG-FLTILE-300 Tf		31	0			31.7	0.1		
6-SPG-FLTILE-3000 T0	2,400	45	0	19.6	Pos.	45	0	19	Pos.
6-SPG-FLTILE-3000 Tf		25.4	0			26	0		
7-SPG-FLTILE-3000 T0	2,400	45	0	20.8	Pos.	45	0	20.8	Pos.
7-SPG-FLTILE-3000 Tf		24.2	0			24.2	0		
8-SPG-FLDBLK-3000 T0	2,400	45	0	25.2	Pos.	42.1	2.6	22.1	Pos.
8-SPG-FLDBLK-3000 Tf		19.8	0			19.9	0		
9-SPG-FLCON-30 T0	24	45	0	17.1	Pos.	45	0	17.1	Pos.
9-SPG-FLCON-30 Tf		27.9	0			27.9	0.1		
10-SPG-FLCON-30 T0	24	45	0	16.6	Pos.	45	0	16.6	Pos.
10-SPG-FLCON-30 Tf		28.4	0.1			28.4	0.1		
11-SPG-STEPS-30 T0	24	45	0	14	Pos.	45	0	14	Pos.
11-SPG-STEPS-30 Tf		31	0.1			31	0		
12-SPG-STEPS-300 T0	240	45	0	20	Pos.	45	0	20.1	Pos.
12-SPG-STEPS-300 Tf		25	0			24.9	0		
13-SPG-FLCON-300 T0	240	45	0	20.8	Pos.	45	0	20.8	Pos.
13-SPG-FLCON-300 Tf		24.2	0			24.2	0		
14-SPG-FLCON-300 T0	240	45	0	20.7	Pos.	45	0	21	Pos.
14-SPG-FLCON-300 Tf		24.3	0.1			24	0		
15-SPG-FLCON-3000 T0	2,400	45	0	21	Pos.	45	0	21.2	Pos.
15-SPG-FLCON-3000 Tf		24	0			23.8	0		
16-SPG-FLCON-3000 T0	2,400	45	0	22.8	Pos.	45	0	23.2	Pos.
16-SPG-FLCON-3000 Tf		22.2	0			21.8	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
1-SPG-GLSWIN-B-S13-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-GLSWIN-B-S13-0 Tf		45	0			45	0		
2-SPG-GLSWIN-B-S14-30 T0	38	45	0	14.2	Pos.	45	0	14.1	Pos.
2-SPG-GLSWIN-B-S14-30 Tf		30.8	0.1			30.9	0.1		
3-SPG-STEPS-B-S11-30 T0	38	45	0	12.9	Pos.	45	0	13.1	Pos.
3-SPG-STEPS-B-S11-30 Tf		32.1	0.1			31.9	0.1		
4-SPG-STEPS-B-S12-300 T0	380	45	0	17.7	Pos.	45	0	17.2	Pos.
4-SPG-STEPS-B-S12-300 Tf		27.3	0.1			27.8	0		
5-SPG-GLSWIN-B-S15-300 T0	380	45	0	25.3	Pos.	45	0	25	Pos.
5-SPG-GLSWIN-B-S15-300 Tf		19.7	0.2			20	0.1		
6-SPG-GLSWIN-B-S16-300 T0	380	45	0	21.8	Pos.	45	0	21.8	Pos.
6-SPG-GLSWIN-B-S16-300 Tf		23.2	0.2			23.2	0.1		
7-SPG-GLSWIN-B-S17-3000 T0	3,800	45	0	25.5	Pos.	43.3	2.9	23.4	Pos.
7-SPG-GLSWIN-B-S17-3000 Tf		19.5	0.1			19.9	0		
8-SPG-GLSWIN-B-S18-3000 T0	3,800	45	0	25.2	Pos.	45	0	25.1	Pos.
8-SPG-GLSWIN-B-S18-3000 Tf		19.8	0			19.9	0		
9-SPG-WLTILE-B-S09-30 T0	38	45	0	17.9	Pos.	45	0	17.2	Pos.
9-SPG-WLTILE-B-S09-30 Tf		27.1	0.1			27.8	0		
10-SPG-WLTILE-B-S10-30 T0	38	45	0	17.3	Pos.	45	0	17	Pos.
10-SPG-WLTILE-B-S10-30 Tf		27.7	0.1			28	0.1		
11-SPG-WLTILE-B-S11-300 T0	380	45	0	22.6	Pos.	45	0	22.5	Pos.
11-SPG-WLTILE-B-S11-300 Tf		22.4	0.1			22.5	0		
12-SPG-WLTILE-B-S12-300 T0	380	45	0	23.9	Pos.	45	0	23.8	Pos.
12-SPG-WLTILE-B-S12-300 Tf		21.1	0			21.2	0		
13-SPG-WLTILE-B-S13-3000 T0	3,800	45	0	23	Pos.	45	0	22.9	Pos.
13-SPG-WLTILE-B-S13-3000 Tf		22	0.1			22.1	0		
14-SPG-WLTILE-B-S14-3000 T0	3,800	45	0	24.1	Pos.	45	0	24.2	Pos.
14-SPG-WLTILE-B-S14-3000 Tf		20.9	0.1			20.8	0		
15-SPG-STEPS-B-S13-3000 T0	3,800	45	0	21.9	Pos.	45	0	21.6	Pos.
15-SPG-STEPS-B-S13-3000 Tf		23.1	0.1			23.4	0		
16-SPG-STEPS-B-S14-3000 T0	3,800	45	0	20.1	Pos.	45	0	20	Pos.
16-SPG-STEPS-B-S14-3000 Tf		24.9	0.1			25	0		
1-SPG-FLDBLK-A-S11-0 T0	0	45	0	7.5	Neg.	45	0	7.5	Neg.
1-SPG-FLDBLK-A-S11-0 Tf		37.5	0.8			37.5	0.4		
2-SPG-GLSPAN-B-S13-0 T0	0	45	0	0	Neg.	45	0	3.4	Neg.
2-SPG-GLSPAN-B-S13-0 Tf		45	0			41.6	3.1		
3-SPG-GLSPAN-B-S14-30 T0	36	45	0	22.1	Pos.	45	0	21.9	Pos.
3-SPG-GLSPAN-B-S14-30 Tf		22.9	0			23.1	0		
4-SPG-FLTILE-B-S16-30 T0	36	45	0	16.2	Pos.	45	0	16.2	Pos.
4-SPG-FLTILE-B-S16-30 Tf		28.8	0.1			28.8	0		
5-SPG-GLSPAN-B-S15-300 T0	360	45	0	26.7	Pos.	45	0	26.7	Pos.
5-SPG-GLSPAN-B-S15-300 Tf		18.3	0.1			18.3	0.1		
6-SPG-GLSPAN-B-S16-300 T0	360	45	0	26	Pos.	45	0	26	Pos.
6-SPG-GLSPAN-B-S16-300 Tf		19	0.1			19	0		
7-SPG-GLSPAN-B-S17-3000 T0	3,600	45	0	24.6	Pos.	45	0	24.4	Pos.
7-SPG-GLSPAN-B-S17-3000 Tf		20.4	0.1			20.6	0.1		
8-SPG-GLSPAN-B-S18-3000 T0	3,600	45	0	24.4	Pos.	45	0	24.5	Pos.
8-SPG-GLSPAN-B-S18-3000 Tf		20.6	0			20.5	0		
9-SPG-EDPAN(B)-B-S13-0 T0	0	44.9	0.2	2	Neg.	43.6	2.4	4.1	Neg.
9-SPG-EDPAN(B)-B-S13-0 Tf		42.9	3.6			39.6	0.5		
10-SPG-EDPAN(B)-B-S14-30 T0	36	45	0	23.1	Pos.	45	0	23	Pos.
10-SPG-EDPAN(B)-B-S14-30 Tf		21.9	0.1			22	0		
11-SPG-EDPAN(B)-B-S15-300 T0	360	45	0	23.9	Pos.	45	0	23.7	Pos.
11-SPG-EDPAN(B)-B-S15-300 Tf		21.1	0			21.3	0.1		
12-SPG-EDPAN(B)-B-S16-300 T0	360	45	0	26	Pos.	45	0	26.1	Pos.
12-SPG-EDPAN(B)-B-S16-300 Tf		19	0			18.9	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
13-SPG-FLTILE-B-S17-300 T0	360	43.3	2.9	20	Pos.	44.7	0.6	21.2	Pos.
13-SPG-FLTILE-B-S17-300 Tf		23.3	0.1			23.4	0		
14-SPG-EDPAN(B)-B-S17-3000 T0	3,600	45	0	25	Pos.	45	0	25	Pos.
14-SPG-EDPAN(B)-B-S17-3000 Tf		20	0.1			20	0		
15-SPG-EDPAN(B)-B-S18-3000 T0	3,600	45	0	28	Pos.	45	0	28.1	Pos.
15-SPG-EDPAN(B)-B-S18-3000 Tf		17	0			16.9	0		
16-SPG-FLTILE-B-S18-3000 T0	3,600	45	0	24	Pos.	45	0	24.2	Pos.
16-SPG-FLTILE-B-S18-3000 Tf		21	0.1			20.8	0		
1-SPG-FLLFIX-B-S13-0 T0	0	45	0	0	Neg.	45	0	1.2	Neg.
1-SPG-FLLFIX-B-S13-0 Tf		45	0			43.8	2.2		
2-SPG-FLLFIX-B-S14-30 T0	26	45	0	16.5	Pos.	45	0	16.3	Pos.
2-SPG-FLLFIX-B-S14-30 Tf		28.5	0.1			28.7	0		
3-SPG-FLLFIX-B-S15-300 T0	260	45	0	21.7	Pos.	45	0	21.3	Pos.
3-SPG-FLLFIX-B-S15-300 Tf		23.3	0.1			23.7	0.1		
4-SPG-FLLFIX-B-S16-300 T0	260	45	0	20	Pos.	45	0	19.8	Pos.
4-SPG-FLLFIX-B-S16-300 Tf		25	0.1			25.2	0		
5-SPG-MCMACH-B-S13-300 T0	260	45	0	17.4	Pos.	45	0	17.2	Pos.
5-SPG-MCMACH-B-S13-300 Tf		27.6	0.1			27.8	0		
6-SPG-FLLFIX-B-S17-3000 T0	2,600	45	0	24.7	Pos.	45	0	24.9	Pos.
6-SPG-FLLFIX-B-S17-3000 Tf		20.3	0			20.1	0		
7-SPG-MCMACH-B-S14-3000 T0	2,600	45	0	23.1	Pos.	45	0	23.1	Pos.
7-SPG-MCMACH-B-S14-3000 Tf		21.9	0.1			21.9	0		
8-SPG-FLLFIX-B-S18-3000 T0	2,600	45	0	20.1	Pos.	45	0	20.1	Pos.
8-SPG-FLLFIX-B-S18-3000 Tf		24.9	0			24.9	0		
9-SPG-OHSIGN-B-S12-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-OHSIGN-B-S12-0 Tf		45	0			45	0		
10-SPG-OHSIGN-B-S13-30 T0	26	45	0	18.6	Pos.	45	0	18.4	Pos.
10-SPG-OHSIGN-B-S13-30 Tf		26.4	0.1			26.6	0		
11-SPG-OHSIGN-B-S14-30 T0	26	45	0	18.4	Pos.	45	0	18.3	Pos.
11-SPG-OHSIGN-B-S14-30 Tf		26.6	0.1			26.7	0		
12-SPG-OHSIGN-B-S15-300 T0	260	45	0	21	Pos.	45	0	21	Pos.
12-SPG-OHSIGN-B-S15-300 Tf		24	0			24	0		
13-SPG-OHSIGN-B-S16-300 T0	260	45	0	20.9	Pos.	45	0	21.1	Pos.
13-SPG-OHSIGN-B-S16-300 Tf		24.1	0.1			23.9	0		
14-SPG-SCGRIL-B-S12-300 T0	260	45	0	21.3	Pos.	45	0	21.7	Pos.
14-SPG-SCGRIL-B-S12-300 Tf		23.7	0			23.3	0.1		
15-SPG-OHSIGN-B-S17-3000 T0	2,600	45	0	22.3	Pos.	45	0	22.3	Pos.
15-SPG-OHSIGN-B-S17-3000 Tf		22.7	0.1			22.7	0		
16-SPG-OHSIGN-B-S18-3000 T0	2,600	45	0	22.5	Pos.	45	0	22.7	Pos.
16-SPG-OHSIGN-B-S18-3000 Tf		22.5	0			22.3	0		
1-SPG-SWCON-A-S13-0 T0	0	45	0	4.6	Neg.	45	0	4.2	Neg.
1-SPG-SWCON-A-S13-0 Tf		40.4	4			40.8	3.4		
2-SPG-SWCON-A-S14-30 T0	33	45	0	13	Pos.	45	0	13.4	Pos.
2-SPG-SWCON-A-S14-30 Tf		32	0.1			31.6	0.1		
3-SPG-SWCON-A-S15-300 T0	330	45	0	21.8	Pos.	45	0	22	Pos.
3-SPG-SWCON-A-S15-300 Tf		23.2	0			23	0		
4-SPG-SWCON-A-S16-300 T0	330	45	0	19.5	Pos.	45	0	19.9	Pos.
4-SPG-SWCON-A-S16-300 Tf		25.5	0			25.1	0		
5-SPG-STGRAT-A-S13-300 T0	330	45	0	17	Pos.	43.4	2.8	15.8	Pos.
5-SPG-STGRAT-A-S13-300 Tf		28	0.1			27.6	0		
6-SPG-STGRAT-A-S14-3000 T0	3,300	45	0	12.8	Pos.	45	0	13.1	Pos.
6-SPG-STGRAT-A-S14-3000 Tf		32.2	0.3			31.9	0		
7-SPG-SWCON-A-S17-3000 T0	3,300	45	0	21.8	Pos.	45	0	22.1	Pos.
7-SPG-SWCON-A-S17-3000 Tf		23.2	0			22.9	0		
8-SPG-SWCON-A-S18-3000 T0	3,300	45	0	24.1	Pos.	45	0	24.4	Pos.
8-SPG-SWCON-A-S18-3000 Tf		20.9	0			20.6	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
9-SPG-EDPAN(A)-A-S12-0 T0	0	45	0	1	Neg.	45	0	1.4	Neg.
9-SPG-EDPAN(A)-A-S12-0 Tf		44	1.8			43.6	2.4		
10-SPG-EDPAN(A)-A-S13-30 T0	33	45	0	20.8	Pos.	45	0	20.8	Pos.
10-SPG-EDPAN(A)-A-S13-30 Tf		24.2	0.1			24.2	0		
11-SPG-EDPAN(A)-A-S14-30 T0	3	45	0	9.9	Pos.	45	0	10.2	Pos.
11-SPG-EDPAN(A)-A-S14-30 Tf		35.1	0.2			34.8	0.1		
12-SPG-EDPAN(A)-A-S15-300 T0	330	45	0	24.6	Pos.	45	0	24.7	Pos.
12-SPG-EDPAN(A)-A-S15-300 Tf		20.4	0.1			20.3	0		
13-SPG-EDPAN(A)-A-S16-300 T0	330	45	0	21.9	Pos.	45	0	22.1	Pos.
13-SPG-EDPAN(A)-A-S16-300 Tf		23.1	0.1			22.9	0		
14-SPG-LABBLANK-300 T0	330	45	0	27	Pos.	45	0	27.1	Pos.
14-SPG-LABBLANK-300 Tf		18	0.1			17.9	0		
15-SPG-EDPAN(A)-A-S17-3000 T0	3,300	45	0	24	Pos.	45	0	24.1	Pos.
15-SPG-EDPAN(A)-A-S17-3000 Tf		21	0			20.9	0		
16-SPG-EDPAN(A)-A-S18-3000 T0	3,300	45	0	26.9	Pos.	45	0	27.1	Pos.
16-SPG-EDPAN(A)-A-S18-3000 Tf		18.1	0			17.9	0		
1-SPG-CWSIGN-A-S13-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-CWSIGN-A-S13-0 Tf		45	0			45	0		
2-SPG-CWSIGN-A-S14-30 T0	37	45	0	20.3	Pos.	45	0	20.3	Pos.
2-SPG-CWSIGN-A-S14-30 Tf		24.7	0.1			24.7	0		
3-SPG-CWSIGN-A-S15-300 T0	370	45	0	18.9	Pos.	45	0	18.8	Pos.
3-SPG-CWSIGN-A-S15-300 Tf		26.1	0.1			26.2	0		
4-SPG-CWSIGN-A-S16-300 T0	370	45	0	18	Pos.	45	0	18.1	Pos.
4-SPG-CWSIGN-A-S16-300 Tf		27	0			26.9	0		
5-SPG-GRNBEN-A-S13-300 T0	370	45	0	21.5	Pos.	45	0	21.4	Pos.
5-SPG-GRNBEN-A-S13-300 Tf		23.5	0.1			23.6	0		
6-SPG-GRNBEN-A-S14-3000 T0	3,700	45	0	21.1	Pos.	45	0	21.3	Pos.
6-SPG-GRNBEN-A-S14-3000 Tf		23.9	0			23.7	0		
7-SPG-CWSIGN-A-S17-3000 T0	3,700	45	0	25.8	Pos.	45	0	25.8	Pos.
7-SPG-CWSIGN-A-S17-3000 Tf		19.2	0			19.2	0		
8-SPG-CWSIGN-A-S18-3000 T0	3,700	45	0	23.1	Pos.	45	0	22.9	Pos.
8-SPG-CWSIGN-A-S18-3000 Tf		21.9	0			22.1	0		
9-SPG-TELEBO-A-S12-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-TELEBO-A-S12-0 Tf		45	0			45	0		
10-SPG-TELEBO-A-S13-30 T0	37	45	0	12.9	Pos.	45	0	13.2	Pos.
10-SPG-TELEBO-A-S13-30 Tf		32.1	0.2			31.8	0		
11-SPG-TELEBO-A-S14-30 T0	37	45	0	23.3	Pos.	45	0	23.2	Pos.
11-SPG-TELEBO-A-S14-30 Tf		21.7	0			21.8	0		
12-SPG-LABBLANK-30 T0	37	45	0	25.7	Pos.	45	0	25.5	Pos.
12-SPG-LABBLANK-30 Tf		19.3	0.1			19.5	0.1		
13-SPG-TELEBO-A-S15-300 T0	370	45	0	21.8	Pos.	45	0	22	Pos.
13-SPG-TELEBO-A-S15-300 Tf		23.2	0.1			23	0		
14-SPG-TELEBO-A-S16-300 T0	370	45	0	23.2	Pos.	45	0	23.5	Pos.
14-SPG-TELEBO-A-S16-300 Tf		21.8	0.1			21.5	0		
15-SPG-TELEBO-A-S17-3000 T0	3,700	45	0	23.4	Pos.	45	0	23.5	Pos.
15-SPG-TELEBO-A-S17-3000 Tf		21.6	0			21.5	0		
16-SPG-TELEBO-A-S18-3000 T0	3,700	45	0	21.3	Pos.	45	0	21.4	Pos.
16-SPG-TELEBO-A-S18-3000 Tf		23.7	0.1			23.6	0		
1-SPG-FLCON-B-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-FLCON-B-S15-0 Tf		45	0			45	0		
2-SPG-STEPS-B-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-STEPS-B-S15-0 Tf		45	0			45	0		
3-SPG-FLCON-B-S16-30 T0	34	45	0	15.6	Pos.	45	0	15.6	Pos.
3-SPG-FLCON-B-S16-30 Tf		29.4	0			29.4	0.1		
4-SPG-STEPS-B-S16-30 T0	34	45	0	14	Pos.	45	0	13.8	Pos.
4-SPG-STEPS-B-S16-30 Tf		31	0.1			31.2	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
5-SPG-FLCON-B-S17-300 T0	340	45	0	16.3	Pos.	45	0	16.5	Pos.
5-SPG-FLCON-B-S17-300 Tf		28.7	0.1			28.5	0.1		
6-SPG-STEPS-B-S17-300 T0	340	45	0	17	Pos.	45	0	16.7	Pos.
6-SPG-STEPS-B-S17-300 Tf		28	0			28.3	0.1		
7-SPG-FLCON-B-S18-3000 T0	3,400	45	0	20.1	Pos.	45	0	20	Pos.
7-SPG-FLCON-B-S18-3000 Tf		24.9	0.1			25	0		
8-SPG-STEPS-B-S18-3000 T0	3,400	45	0	19.4	Pos.	45	0	19.6	Pos.
8-SPG-STEPS-B-S18-3000 Tf		25.6	0			25.4	0		
9-SPG-SCGRIL-B-S13-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-SCGRIL-B-S13-0 Tf		45	0			45	0		
10-SPG-LABBLANK-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
10-SPG-LABBLANK-0 Tf		45	0			45	0		
11-SPG-LABBLANK-30 T0	34	45	0	25.3	Pos.	45	0	25.2	Pos.
11-SPG-LABBLANK-30 Tf		19.7	0.1			19.8	0		
12-SPG-SCGRIL-B-S14-30 T0	34	45	0	13.6	Pos.	45	0	13.5	Pos.
12-SPG-SCGRIL-B-S14-30 Tf		31.4	0.1			31.5	0.1		
13-SPG-SCGRIL-B-S15-30 T0	34	45	0	10.9	Pos.	45	0	11.1	Pos.
13-SPG-SCGRIL-B-S15-30 Tf		34.1	0.2			33.9	0.1		
14-SPG-SCGRIL-B-S16-300 T0	340	45	0	19.1	Pos.	45	0	19.2	Pos.
14-SPG-SCGRIL-B-S16-300 Tf		25.9	0			25.8	0		
15-SPG-SCGRIL-B-S17-3000 T0	3,400	45	0	18.4	Pos.	45	0	18.1	Pos.
15-SPG-SCGRIL-B-S17-3000 Tf		26.6	0.1			26.9	0		
16-SPG-SCGRIL-B-S18-3000 T0	3,400	45	0	25.2	Pos.	45	0	25.6	Pos.
16-SPG-SCGRIL-B-S18-3000 Tf		19.8	0			19.4	0		
1-SPG-WLTILE-B-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-WLTILE-B-S15-0 Tf		45	0			45	0		
2-SPG-MCMACH-B-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-MCMACH-B-S15-0 Tf		45	0			45	0		
3-SPG-WLTILE-B-S16-30 T0	24	45	0	14.7	Pos.	45	0	14.2	Pos.
3-SPG-WLTILE-B-S16-30 Tf		30.3	0.2			30.8	0.1		
4-SPG-MCMACH-B-S16-30 T0	24	45	0	19.2	Pos.	45	0	19.3	Pos.
4-SPG-MCMACH-B-S16-30 Tf		25.8	0.1			25.7	0.1		
5-SPG-WLTILE-B-S17-300 T0	240	45	0	18.6	Pos.	45	0	18.3	Pos.
5-SPG-WLTILE-B-S17-300 Tf		26.4	0.1			26.7	0		
6-SPG-MCMACH-B-S17-300 T0	240	45	0	19.6	Pos.	45	0	19.4	Pos.
6-SPG-MCMACH-B-S17-300 Tf		25.4	0.1			25.6	0		
7-SPG-WLTILE-B-S18-3000 T0	2,400	45	0	19.6	Pos.	45	0	19	Pos.
7-SPG-WLTILE-B-S18-3000 Tf		25.4	0.1			26	0		
8-SPG-MCMACH-B-S18-3000 T0	2,400	45	0	23	Pos.	45	0	23	Pos.
8-SPG-MCMACH-B-S18-3000 Tf		22	0			22	0		
9-SPG-FLDBLK-A-S12-30 T0	24	45	0	25.2	Pos.	45	0	24.8	Pos.
9-SPG-FLDBLK-A-S12-30 Tf		19.8	0.1			20.2	0.1		
10-SPG-CWPNTD-A-S16-30 T0	24	45	0	15	Pos.	45	0	14.8	Pos.
10-SPG-CWPNTD-A-S16-30 Tf		30	0			30.2	0		
11-SPG-FLDBLK-A-S13-300 T0	240	45	0	26.1	Pos.	45	0	25.7	Pos.
11-SPG-FLDBLK-A-S13-300 Tf		18.9	0			19.3	0		
12-SPG-CWPNTD-A-S17-300 T0	240	45	0	19	Pos.	45	0	18.7	Pos.
12-SPG-CWPNTD-A-S17-300 Tf		26	0.1			26.3	0		
13-SPG-LABBLANK-300 T0	240	45	0	25.4	Pos.	45	0	25.1	Pos.
13-SPG-LABBLANK-300 Tf		19.6	0.1			19.9	0		
14-SPG-FLDBLK-A-S14-3000 T0	2,400	45	0	25	Pos.	45	0	24.9	Pos.
14-SPG-FLDBLK-A-S14-3000 Tf		20	0			20.1	0		
15-SPG-CWPNTD-A-S18-3000 T0	2,400	45	0	19.2	Pos.	45	0	18.6	Pos.
15-SPG-CWPNTD-A-S18-3000 Tf		25.8	0			26.4	0.1		
16-SPG-LABBLANK-3000 T0	2,400	45	0	25.2	Pos.	45	0	25.1	Pos.
16-SPG-LABBLANK-3000 Tf		19.8	0			19.9	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Std Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Std Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
1-SPG-FLDBLK-A-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-SPG-FLDBLK-A-S15-0 Tf		45	0			45	0		
2-SPG-STGRAT-A-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-SPG-STGRAT-A-S15-0 Tf		45	0			45	0		
3-SPG-FLDBLK-A-S16-30 T0	31	45	0	26.8	Pos.	45	0	26.4	Pos.
3-SPG-FLDBLK-A-S16-30 Tf		18.2	0			18.6	0		
4-SPG-STGRAT-A-S16-30 T0	31	45	0	10.5	Pos.	45	0	11	Pos.
4-SPG-STGRAT-A-S16-30 Tf		34.5	0.2			34	0		
5-SPG-FLDBLK-A-S17-300 T0	310	45	0	25.5	Pos.	45	0	25.2	Pos.
5-SPG-FLDBLK-A-S17-300 Tf		19.5	0.1			19.8	0		
6-SPG-STGRAT-A-S17-300 T0	310	45	0	14.7	Pos.	45	0	15.3	Pos.
6-SPG-STGRAT-A-S17-300 Tf		30.3	0.1			29.7	0.1		
7-SPG-FLDBLK-A-S18-3000 T0	3,100	45	0	26.5	Pos.	45	0	26.2	Pos.
7-SPG-FLDBLK-A-S18-3000 Tf		18.5	0			18.8	0		
8-SPG-STGRAT-A-S18-3000 T0	3,100	45	0	16.2	Pos.	45	0	18.3	Pos.
8-SPG-STGRAT-A-S18-3000 Tf		28.8	3			26.7	0.2		
9-SPG-GRNBEN-A-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-SPG-GRNBEN-A-S15-0 Tf		45	0			45	0		
10-SPG-WLTILE-B-S19-0 T0	0	45	0	0	Neg.	45	0	0.1	Neg.
10-SPG-WLTILE-B-S19-0 Tf		45	0			44.9	0.2		
11-SPG-GRNBEN-A-S16-30 T0	31	45	0	20.2	Pos.	43.6	2.4	18.7	Pos.
11-SPG-GRNBEN-A-S16-30 Tf		24.8	0.1			24.9	0		
12-SPG-LABBLK-30 T0	31	45	0	26	Pos.	45	0	25.6	Pos.
12-SPG-LABBLK-30 Tf		19	0			19.4	0		
13-SPG-GRNBEN-A-S17-300 T0	310	45	0	18.1	Pos.	45	0	18.1	Pos.
13-SPG-GRNBEN-A-S17-300 Tf		26.9	0.1			26.9	0		
14-SPG-LABBLK-300 T0	310	45	0	26	Pos.	45	0	25.7	Pos.
14-SPG-LABBLK-300 Tf		19	0			19.3	0		
15-SPG-GRNBEN-A-S18-3000 T0	3,100	45	0	24.1	Pos.	45	0	24.1	Pos.
15-SPG-GRNBEN-A-S18-3000 Tf		20.9	0			20.9	0		
16-SPG-LABBLK-3000 T0	3,100	45	0	26.2	Pos.	43.4	2.7	24.4	Pos.
16-SPG-LABBLK-3000 Tf		18.8	0			19	0		

APPENDIX Q: CULTURE RESULTS FOR VCF SAMPLES USING SHEEP BLOOD AGAR MEDIUM

Vacuum Cassette Filters Floor Concrete

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-VCF-FLCON-B-S01-0	0.0E+00	11	1.0	5	5.0	55	#DIV/0!
	0.0E+00	11	2.9	0	0.0	0	#DIV/0!
1-VCF-FLCON-B-S11-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.8	0	0.0	0	#DIV/0!
1-VCF-FLCON-B-S15-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.6	0	0.0	0	#DIV/0!
2-VCF-FLCON-B-S02-30	2.7E+01	11	1.0	4	4.0	44	163.0
	2.7E+01	11	2.9	9	3.1	34	126.4
1-VCF-FLCON-B-S05-30	2.9E+01	11	1.0	1	1.0	11	37.9
	2.9E+01	11	2.7	2	0.7	8	28.1
2-VCF-FLCON-B-S06-30	2.9E+01	11	1.0	2	2.0	22	75.9
	2.9E+01	11	3.0	1	0.3	4	12.6
3-VCF-FLCON-B-S12-30	5.1E+01	11	1.0	1	1.0	11	21.6
	5.1E+01	11	2.5	0	0.0	0	0.0
3-VCF-FLCON-B-S16-30	2.9E+01	11	1.0	0	0.0	0	0.0
	2.9E+01	11	2.5	0	0.0	0	0.0
5-VCF-FLCON-B-S03-300	2.7E+02	11	1.0	6	6.0	66	24.4
	2.7E+02	11	4.0	8	2.0	22	8.1
5-VCF-FLCON-B-S07-300	2.9E+02	11	1.0	0	0.0	0	0.0
	2.9E+02	11	2.7	0	0.0	0	0.0
6-VCF-FLCON-B-S08-300	2.9E+02	11	1.0	0	0.0	0	0.0
	2.9E+02	11	2.6	2	0.8	8	2.9
5-VCF-FLCON-B-S13-300	5.1E+02	11	1.0	0	0.0	0	0.0
	5.1E+02	11	2.5	0	0.0	0	0.0
5-VCF-FLCON-B-S17-300	2.9E+02	11	1.0	0	0.0	0	0.0
	2.9E+02	11	2.7	0	0.0	0	0.0
7-VCF-FLCON-B-S04-3,000	2.7E+03	11	1.0	111	111.0	1221	45.2
	2.7E+03	11	2.8	232	82.9	911	33.8
7-VCF-FLCON-B-S09-3,000	2.9E+03	11	1.0	5	5.0	55	1.9
	2.9E+03	11	2.7	6	2.2	24	0.8
8-VCF-FLCON-B-S10-3,000	2.9E+03	11	1.0	1	1.0	11	0.4
	2.9E+03	11	2.5	3	1.2	13	0.5
7-VCF-FLCON-B-S14-3,000	5.1E+03	11	1.0	10	10.0	110	2.2
	5.1E+03	11	2.9	26	9.0	99	1.9
7-VCF-FLCON-B-S18-3,000	2.9E+03	11	1.0	3	3.0	33	1.1
	2.9E+03	11	2.7	3	1.1	12	0.4

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Vacuum Cassette Filters Steps (with Metal Grid)

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-VCF-STEPS-B-S01-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.4	0	0.0	0	#DIV/0!
2-VCF-STEPS-B-S11-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	1.6	0	0.0	0	#DIV/0!
2-VCF-STEPS-B-S15-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.4	0	0.0	0	#DIV/0!
3-VCF-STEPS-B-S02-30	2.1E+01	11	1.0	4	4.0	44	209.5
	2.1E+01	11	2.8	1	0.4	4	18.7
3-VCF-STEPS-B-S05-30	2.9E+01	11	1.0	6	6.0	66	227.6
	2.9E+01	11	2.4	5	2.1	23	79.0
4-VCF-STEPS-B-S06-30	2.9E+01	11	1.0	12	12.0	132	455.2
	2.9E+01	11	1.7	23	13.5	149	513.2
4-VCF-STEPS-B-S12-30	5.1E+01	11	1.0	0	0.0	0	0.0
	5.1E+01	11	2.0	0	0.0	0	0.0
4-VCF-STEPS-B-S16-30	2.9E+01	11	1.0	0	0.0	0	0.0
	2.9E+01	11	2.3	0	0.0	0	0.0
5-VCF-STEPS-B-S03-300	2.1E+02	11	1.0	0	0.0	0	0.0
	2.1E+02	11	1.8	0	0.0	0	0.0
12-VCF-STEPS-B-S07-300	2.9E+02	11	1.0	31	31.0	341	117.6
	2.9E+02	11	2.2	40	18.2	200	69.0
13-VCF-STEPS-B-S08-300	2.9E+02	11	1.0	9	9.0	99	34.1
	2.9E+02	11	2.2	0	0.0	0	0.0
6-VCF-STEPS-B-S13-300	5.1E+02	11	1.0	0	0.0	0	0.0
	5.1E+02	11	2.4	3	1.3	14	2.7
6-VCF-STEPS-B-S17-300	2.9E+02	11	1.0	0	0.0	0	0.0
	2.9E+02	11	2.3	0	0.0	0	0.0
7-VCF-STEPS-B-S04-3,000	2.1E+03	11	1.0	0	0.0	0	0.0
	2.1E+03	11	2.4	0	0.0	0	0.0
14-VCF-STEPS-B-S09-3,000	2.9E+03	11	1.0	0	0.0	0	0.0
	2.9E+03	11	2.4	0	0.0	0	0.0
15-VCF-STEPS-B-S10-3,000	2.9E+03	11	1.0	2	2.0	22	0.8
	2.9E+03	11	2.6	0	0.0	0	0.0
8-VCF-STEPS-B-S14-3,000	5.1E+03	11	1.0	0	0.0	0	0.0
	5.1E+03	11	2.3	0	0.0	0	0.0
8-VCF-STEPS-B-S18-3,000	2.9E+03	11	1.0	1	1.0	11	0.4
	2.9E+03	11	2.3	0	0.0	0	0.0

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Vacuum Cassette Filters Carpet/Rug

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
9-VCF-CARPET-A-S04-0	0.0E+00	11	1.0	1	1.0	11	#DIV/0!
	0.0E+00	11	2.5	0	0.0	0	#DIV/0!
9-VCF-CARPET-A-S08-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.5	0	0.0	0	#DIV/0!
9-VCF-CARPET-A-S12-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.4	0	0.0	0	#DIV/0!
3-VCF-CARPET-A-S01-30	2.7E+01	11	1.0	0	0.0	0	0.0
	2.7E+01	11	2.0	0	0.0	0	0.0
10-VCF-CARPET-A-S05-30	2.9E+01	11	1.0	3	3.0	33	113.8
	2.9E+01	11	2.6	2	0.8	8	29.2
11-VCF-CARPET-A-S09-30	2.6E+01	11	1.0	0	0.0	0	0.0
	2.6E+01	11	2.2	0	0.0	0	0.0
11-VCF-CARPET-A-S13-30	5.1E+01	11	1.0	0	0.0	0	0.0
	5.1E+01	11	2.4	0	0.0	0	0.0
4-VCF-CARPET-A-S16-30	3.0E+01	11	1.0	0	0.0	0	0.0
	3.0E+01	11	2.1	0	0.0	0	0.0
6-VCF-CARPET-A-S02-300	2.7E+02	11	1.0	0	0.0	0	0.0
	2.7E+02	11	0.5	5	10.0	110	40.7
11-VCF-CARPET-A-S06-300	2.9E+02	11	1.0	9	9.0	99	34.1
	2.9E+02	11	2.6	0	0.0	0	0.0
13-VCF-CARPET-A-S10-300	2.6E+02	11	1.0	2	2.0	22	8.5
	2.6E+02	11	2.5	0	0.0	0	0.0
13-VCF-CARPET-A-S14-300	5.1E+02	11	1.0	0	0.0	0	0.0
	5.1E+02	11	2.3	0	0.0	0	0.0
11-VCF-CARPET-A-S17-300	3.0E+02	11	1.0	1	1.0	11	3.7
	3.0E+02	11	2.8	0	0.0	0	0.0
8-VCF-CARPET-A-S03-3,000	2.7E+03	11	1.0	0	0.0	0	0.0
	2.7E+03	11	2.5	0	0.0	0	0.0
16-VCF-CARPET-A-S07-3,000	2.9E+03	11	1.0	0	0.0	0	0.0
	2.9E+03	11	2.6	0	0.0	0	0.0
15-VCF-CARPET-A-S11-3,000	2.6E+03	11	1.0	1	1.0	11	0.4
	2.6E+03	11	2.9	0	0.0	0	0.0
15-VCF-CARPET-A-S15-3,000	5.1E+03	11	1.0	4	4.0	44	0.9
	5.1E+03	11	2.1	2	1.0	10	0.2
14-VCF-CARPET-A-S18-3,000	3.0E+03	11	1.0	0	0.0	0	0.0
	3.0E+03	11	2.7	0	0.0	0	0.0

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Vacuum Cassette Filters Subway Car HVAC Filter

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-VCF-SCFILT-B-S04-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	1.4	0	0.0	0	#DIV/0!
2-VCF-SCFILT-B-S05-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	0.7	0	0.0	0	#DIV/0!
10-VCF-SCFILT-B-S12-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	0.1	0	0.0	0	#DIV/0!
4-VCF-SCFILT-B-S01-30	2.7E+01	11	1.0	7	7.0	77	285.2
	2.7E+01	11	0.8	0	0.0	0	0.0
3-VCF-SCFILT-B-S06-30	3.2E+01	11	1.0	0	0.0	0	0.0
	3.2E+01	11	1.4	0	0.0	0	0.0
4-VCF-SCFILT-B-S07-30	3.2E+01	11	1.0	0	0.0	0	0.0
	3.2E+01	11	0.2	2	10.0	110	343.8
12-VCF-SCFILT-B-S13-30	5.1E+01	11	1.0	0	0.0	0	0.0
	5.1E+01	11	0.2	1	5.0	55	107.8
9-VCF-SCFILT-B-S16-30	3.0E+01	11	1.0	0	0.0	0	0.0
	3.0E+01	11	0.3	0	0.0	0	0.0
11-VCF-SCFILT-B-S02-300	2.7E+02	11	1.0	0	0.0	0	0.0
	2.7E+02	11	1.8	0	0.0	0	0.0
5-VCF-SCFILT-B-S08-300	3.2E+02	11	1.0	0	0.0	0	0.0
	3.2E+02	11	0.1	2	20.0	220	68.8
6-VCF-SCFILT-B-S09-300	3.2E+02	11	1.0	0	0.0	0	0.0
	3.2E+02	11	0.1	1	10.0	110	34.4
14-VCF-SCFILT-B-S14-300	5.1E+02	11	1.0	0	0.0	0	0.0
	5.1E+02	11	0.2	0	0.0	0	0.0
12-VCF-SCFILT-B-S17-300	3.0E+02	11	1.0	0	0.0	0	0.0
	3.0E+02	11	0.3	0	0.0	0	0.0
14-VCF-SCFILT-B-S03-3000	2.7E+03	11	1.0	0	0.0	0	0.0
	2.7E+03	11	1.5	0	0.0	0	0.0
7-VCF-SCFILT-B-S10-3,000	3.2E+03	11	1.0	1	1.0	11	0.3
	3.2E+03	11	0.9	2	2.2	24	0.8
8-VCF-SCFILT-B-S11-3,000	3.2E+03	11	1.0	0	0.0	0	0.0
	3.2E+03	11	0.3	0	0.0	0	0.0
16-VCF-SCFILT-B-S15-3,000	5.1E+03	11	1.0	0	0.0	0	0.0
	5.1E+03	11	0.2	0	0.0	0	0.0
15-VCF-SCFILT-B-S18-3,000	3.0E+03	11	1.0	0	0.0	0	0.0
	3.0E+03	11	0.3	1	3.3	37	1.2
Use values highlighted in green for reporting.							
Previously called UD for undetected 3/27/2019 scn.							

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Vacuum Cassette Filters Sidewalk Concrete

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
2-VCF-SWCON-A-S01-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.5	0	0.0	0	#DIV/0!
9-VCF-SWCON-A-S05-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	3.0	0	0.0	0	#DIV/0!
9-VCF-SWCON-A-S15-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.9	0	0.0	0	#DIV/0!
4-VCF-SWCON-A-S02-30	2.1E+01	11	1.0	0	0.0	0	0.0
	2.1E+01	11	2.4	0	0.0	0	0.0
10-VCF-SWCON-A-S06-30	3.2E+01	11	1.0	0	0.0	0	0.0
	3.2E+01	11	2.8	2	0.7	8	24.6
11-VCF-SWCON-A-S07-30	3.2E+01	11	1.0	1	1.0	11	34.4
	3.2E+01	11	2.6	0	0.0	0	0.0
10-VCF-SWCON-A-S12-30	3.0E+01	11	1.0	0	0.0	0	0.0
	3.0E+01	11	3.0	0	0.0	0	0.0
11-VCF-SWCON-A-S16-30	2.9E+01	11	1.0	0	0.0	0	0.0
	2.9E+01	11	2.9	0	0.0	0	0.0
6-VCF-SWCON-A-S03-300	2.1E+02	11	1.0	0	0.0	0	0.0
	2.1E+02	11	2.5	0	0.0	0	0.0
12-VCF-SWCON-A-S08-300	3.2E+02	11	1.0	0	0.0	0	0.0
	3.2E+02	11	2.9	0	0.0	0	0.0
13-VCF-SWCON-A-S09-300	3.2E+02	11	1.0	1	1.0	11	3.4
	3.2E+02	11	2.8	0	0.0	0	0.0
13-VCF-SWCON-A-S13-300	3.0E+02	11	1.0	0	0.0	0	0.0
	3.0E+02	11	3.1	0	0.0	0	0.0
13-VCF-SWCON-A-S17-300	2.9E+02	11	1.0	0	0.0	0	0.0
	2.9E+02	11	2.8	1	0.4	4	1.4
8-VCF-SWCON-A-S04-3,000	2.1E+03	11	1.0	1	1.0	11	0.5
	2.1E+03	11	1.4	3	2.1	24	1.1
14-VCF-SWCON-A-S10-3,000	3.2E+03	11	1.0	1	1.0	11	0.3
	3.2E+03	11	2.9	4	1.4	15	0.5
15-VCF-SWCON-A-S11-3,000	3.2E+03	11	1.0	2	2.0	22	0.7
	3.2E+03	11	2.7	3	1.1	12	0.4
16-VCF-SWCON-A-S14-3,000	3.0E+03	11	1.0	1	1.0	11	0.4
	3.0E+03	11	2.9	5	1.7	19	0.6
15-VCF-SWCON-A-S18-3,000	2.9E+03	11	1.0	8	8.0	88	3.0
	2.9E+03	11	2.9	17	5.9	64	2.2

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Vacuum Cassette Filters Pavement (Asphalt)

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
1-VCF-PAVEMT-A-S04-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.3	0	0.0	0	#DIV/0!
2-VCF-PAVEMT-A-S05-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	1.8	0	0.0	0	#DIV/0!
10-VCF-PAVEMT-A-S15-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.6	0	0.0	0	#DIV/0!
9-VCF-PAVEMT-A-S01-30	2.7E+01	11	1.0	5	5.0	55	203.7
	2.7E+01	11	2.8	25	8.9	98	363.8
3-VCF-PAVEMT-A-S06-30	2.6E+01	11	1.0	0	0.0	0	0.0
	2.6E+01	11	2.6	0	0.0	0	0.0
4-VCF-PAVEMT-A-S07-30	2.6E+01	11	1.0	0	0.0	0	0.0
	2.6E+01	11	2.3	0	0.0	0	0.0
3-VCF-PAVEMT-A-S12-30	3.0E+01	11	1.0	0	0.0	0	0.0
	3.0E+01	11	2.5	0	0.0	0	0.0
12-VCF-PAVEMT-A-S16-30	2.9E+01	11	1.0	0	0.0	0	0.0
	2.9E+01	11	2.7	0	0.0	0	0.0
12-VCF-PAVEMT-A-S02-300	2.7E+02	11	1.0	23	23.0	253	93.7
	2.7E+02	11	2.9	0	0.0	0	0.0
5-VCF-PAVEMT-A-S08-300	2.6E+02	11	1.0	1	1.0	11	4.2
	2.6E+02	11	2.5	0	0.0	0	0.0
6-VCF-PAVEMT-A-S09-300	2.6E+02	11	1.0	0	0.0	0	0.0
	2.6E+02	11	2.8	0	0.0	0	0.0
6-VCF-PAVEMT-A-S13-300	3.0E+02	11	1.0	1	1.0	11	3.7
	3.0E+02	11	2.7	0	0.0	0	0.0
14-VCF-PAVEMT-A-S17-300	2.9E+02	11	1.0	0	0.0	0	0.0
	2.9E+02	11	2.7	0	0.0	0	0.0
15-VCF-PAVEMT-A-S03-3000	2.7E+03	11	1.0	0	0.0	0	0.0
	2.7E+03	11	3.1	0	0.0	0	0.0
7-VCF-PAVEMT-A-S10-3,000	2.6E+03	11	1.0	3	3.0	33	1.3
	2.6E+03	11	2.2	1	0.5	5	0.2
8-VCF-PAVEMT-A-S11-3,000	2.6E+03	11	1.0	1	1.0	11	0.4
	2.6E+03	11	3.1	7	2.3	25	1.0
8-VCF-PAVEMT-A-S14-3,000	3.0E+03	11	1.0	6	6.0	66	2.2
	3.0E+03	11	2.8	5	1.8	20	0.7
16-VCF-PAVEMT-A-S18-3,000	2.9E+03	11	1.0	1	1.0	11	0.4
	2.9E+03	11	2.8	0	0.0	0	0.0

Use values highlighted in green for reporting.

Previously called UD for undetected 3/27/2019 scn.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

Vacuum Cassette Filters Field Blank

Sample ID	Spore Load[1]	Extraction Volume (mL)	Volume in Filter Cup (mL)	Plate Counts (CFU)	Average Sample Concentration		Percent Recovery
					CFU/mL	Total CFU	
10-VCF-FLDBLK-A-S04-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	3.2	0	0.0	0	#DIV/0!
1-VCF-FLDBLK-A-S08-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	3.0	0	0.0	0	#DIV/0!
1-VCF-FLDBLK-A-S12-0	0.0E+00	11	1.0	0	0.0	0	#DIV/0!
	0.0E+00	11	2.9	0	0.0	0	#DIV/0!
10-VCF-FLDBLK-A-S01-30	2.7E+01	11	1.0	0	0.0	0	0.0
	2.7E+01	11	3.0	0	0.0	0	0.0
12-VCF-FLDBLK-A-S05-30	2.6E+01	11	1.0	0	0.0	0	0.0
	2.6E+01	11	2.9	0	0.0	0	0.0
2-VCF-FLDBLK-A-S09-30	3.0E+01	11	1.0	0	0.0	0	0.0
	3.0E+01	11	3.0	0	0.0	0	0.0
2-VCF-FLDBLK-A-S13-30	1.9E+01	11	1.0	0	0.0	0	0.0
	1.9E+01	11	3.0	0	0.0	0	0.0
3-VCF-FLDBLK-A-S14-30	1.9E+01	11	1.0	0	0.0	0	0.0
	1.9E+01	11	3.0	0	0.0	0	0.0
13-VCF-FLDBLK-A-S02-300	2.7E+02	11	1.0	0	0.0	0	0.0
	2.7E+02	11	3.0	0	0.0	0	0.0
14-VCF-FLDBLK-A-S06-300	2.6E+02	11	1.0	0	0.0	0	0.0
	2.6E+02	11	2.9	0	0.0	0	0.0
5-VCF-FLDBLK-A-S10-300	3.0E+02	11	1.0	1	1.0	11	3.7
	3.0E+02	11	2.6	0	0.0	0	0.0
4-VCF-FLDBLK-A-S15-300	1.9E+02	11	1.0	2	2.0	22	11.6
	1.9E+02	11	2.9	0	0.0	0	0.0
5-VCF-FLDBLK-A-S16-300	1.9E+02	11	1.0	0	0.0	0	0.0
	1.9E+02	11	2.9	2	0.7	8	4.0
16-VCF-FLDBLK-A-S03-3000	2.7E+03	11	1.0	2	2.0	22	0.8
	2.7E+03	11	3.0	2	0.7	7	0.3
16-VCF-FLDBLK-A-S07-3,000	2.6E+03	11	1.0	1	1.0	11	0.4
	2.6E+03	11	2.5	0	0.0	0	0.0
7-VCF-FLDBLK-A-S11-3,000	3.0E+03	11	1.0	0	0.0	0	0.0
	3.0E+03	11	2.8	0	0.0	0	0.0
6-VCF-FLDBLK-A-S17-3000	1.9E+03	11	1.0	0	0.0	0	0.0
	1.9E+03	11	3.0	4	1.3	15	0.8
7-VCF-FLDBLK-A-S18-3000	1.9E+03	11	1.0	0	0.0	0	0.0
	1.9E+03	11	3.0	3	1.0	11	0.6

Use values highlighted in green for reporting.

[1] Actual number of B. a. Sterne spores spiked onto filter based on spiking suspension titer and volume of suspension applied.

APPENDIX R: RV-PCR RESULTS FOR VCF SAMPLES USING CHROMOSOMAL AND pXO1 GENE TARGETS

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
1-VCF-STEPS-B-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-VCF-STEPS-B-S01-0 Tf		45	0			45	0		
2-VCF-SWCON-A-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-VCF-SWCON-A-S01-0 Tf		45	0			45	0		
3-VCF-STEPS-B-S02-30 T0	21	45	0	0	Neg.	45	0	0	Neg.
3-VCF-STEPS-B-S02-30 Tf		45	0			45	0		
4-VCF-SWCON-A-S02-30 T0	21	45	0	0	Neg.	45	0	0	Neg.
4-VCF-SWCON-A-S02-30 Tf		45	0			45	0		
5-VCF-STEPS-B-S03-300 T0	210	45	0	0	Neg.	45	0	1.1	Neg.
5-VCF-STEPS-B-S03-300 Tf		45	0			43.9	1.9		
6-VCF-SWCON-A-S03-300 T0	210	45	0	11.9	Pos.	44.2	1.3	11.2	Pos.
6-VCF-SWCON-A-S03-300 Tf		33.1	0.1			33	0.1		
7-VCF-STEPS-B-S04-3,000 T0	2,100	45	0	12.1	Pos.	45	0	12.2	Pos.
7-VCF-STEPS-B-S04-3,000 Tf		32.9	0.1			32.8	0.1		
8-VCF-SWCON-A-S04-3,000 T0	2,100	45	0	17.2	Pos.	44.6	0.6	17.3	Pos.
8-VCF-SWCON-A-S04-3,000 Tf		27.8	0.1			27.3	0		
1-VCF-FLCON-B-S01-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-VCF-FLCON-B-S01-0 Tf		45	0			45	0		
2-VCF-FLCON-B-S02-30 T0	27	45	0	0	Neg.	45	0	1	Neg.
2-VCF-FLCON-B-S02-30 Tf		45	0			44	1.8		
3-VCF-CARPET-A-S01-30 T0	27	45	0	0	Neg.	45	0	0	Neg.
3-VCF-CARPET-A-S01-30 Tf		45	0			45	0		
4-VCF-SCFILT-B-S01-30 T0	27	45	0	0	Neg.	45	0	0	Neg.
4-VCF-SCFILT-B-S01-30 Tf		45	0			45	0		
5-VCF-FLCON-B-S03-300 T0	270	45	0	18.6	Pos.	45	0	19	Pos.
5-VCF-FLCON-B-S03-300 Tf		26.4	0.1			26	0		
6-VCF-CARPET-A-S02-300 T0	270	45	0	14.4	Pos.	43.5	1.7	13.1	Pos.
6-VCF-CARPET-A-S02-300 Tf		30.6	0.1			30.4	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
7-VCF-FLCON-B-S04-3,000 T0	2,700	45	0	14.3	Pos.	45	0	14.6	Pos.
7-VCF-FLCON-B-S04-3,000 Tf		30.7	0.1			30.4	0.1		
8-VCF-CARPET-A-S03-3,000 T0	2,700	45	0	20.2	Pos.	45	0	20.5	Pos.
8-VCF-CARPET-A-S03-3,000 Tf		24.8	0.1			24.5	0.1		
9-VCF-PAVEMT-A-S01-30 T0	27	45	0	0	Neg.	45	0	0	Neg.
9-VCF-PAVEMT-A-S01-30 Tf		45	0			45	0		
10-VCF-FLDBLK-A-S01-30 T0	27	45	0	0	Neg.	45	0	0	Neg.
10-VCF-FLDBLK-A-S01-30 Tf		45	0			45	0		
11-VCF-SCFILT-B-S02-300 T0	270	45	0	0	Neg.	45	0	0	Neg.
11-VCF-SCFILT-B-S02-300 Tf		45	0			45	0		
12-VCF-PAVEMT-A-S02-300 T0	270	45	0	0	Neg.	45	0	0	Neg.
12-VCF-PAVEMT-A-S02-300 Tf		45	0			45	0		
13-VCF-FLDBLK-A-S02-300 T0	270	45	0	0	Neg.	43.8	2.1	-1.2	Neg.
13-VCF-FLDBLK-A-S02-300 Tf		45	0			45	0		
14-VCF-SCFILT-B-S03-3000 T0	2,700	45	0	13.2	Pos.	44.4	1	13	Pos.
14-VCF-SCFILT-B-S03-3000 Tf		31.8	0			31.4	0.1		
15-VCF-PAVEMT-A-S03-3000 T0	2,700	45	0	0.9	Neg.*	45	0	5.3	Neg.*
15-VCF-PAVEMT-A-S03-3000 Tf		44.1	1.5			39.7	2.3		
16-VCF-FLDBLK-A-S03-3000 T0	2,700	45	0	25.9	Pos.	44	1.8	24.8	Pos.
16-VCF-FLDBLK-A-S03-3000 Tf		19.1	0			19.1	0		
1-VCF-FLCON-B-S05-30 T0	29	45	0	0	Neg.	45	0	0.7	Neg.
1-VCF-FLCON-B-S05-30 Tf		45	0			44.3	1.2		
2-VCF-FLCON-B-S06-30 T0	29	45	0	0	Neg.	44.3	1.2	-0.7	Neg.
2-VCF-FLCON-B-S06-30 Tf		45	0			45	0		
3-VCF-STEPS-B-S05-30 T0	29	45	0	0	Neg.	45	0	0	Neg.
3-VCF-STEPS-B-S05-30 Tf		45	0			45	0		
4-VCF-STEPS-B-S06-30 T0	29	45	0	12.7	Pos.	45	0	12.8	Pos.
4-VCF-STEPS-B-S06-30 Tf		32.3	0.1			32.2	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
5-VCF-FLCON-B-S07-300 T0	290	45	0	0	Neg.	45	0	0	Neg.
5-VCF-FLCON-B-S07-300 Tf		45	0			45	0		
6-VCF-FLCON-B-S08-300 T0	290	45	0	17.9	Pos.	45	0	18	Pos.
6-VCF-FLCON-B-S08-300 Tf		27.1	0			27	0		
7-VCF-FLCON-B-S09-3,000 T0	2,900	45	0	N/A*	N/A*	45	0	N/A*	N/A*
7-VCF-FLCON-B-S09-3,000 Tf		N/A*	N/A*			N/A*	N/A*		
8-VCF-FLCON-B-S10-3,000 T0	2,900	45	0	16.9	Pos.	45	0	17	Pos.
8-VCF-FLCON-B-S10-3,000 Tf		28.1	0			28	0		
9-VCF-CARPET-A-S04-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-VCF-CARPET-A-S04-0 Tf		45	0			45	0		
10-VCF-CARPET-A-S05-30 T0	29	45	0	9.3	Pos.	45	0	9.2	Pos.
10-VCF-CARPET-A-S05-30 Tf		35.7	0.2			35.8	0.3		
11-VCF-CARPET-A-S06-300 T0	290	45	0	14.2	Pos.	45	0	14.1	Pos.
11-VCF-CARPET-A-S06-300 Tf		30.8	0.2			30.9	0.1		
12-VCF-STEPS-B-S07-300 T0	290	45	0	18.4	Pos.	45	0	18.4	Pos.
12-VCF-STEPS-B-S07-300 Tf		26.6	0.1			26.6	0		
13-VCF-STEPS-B-S08-300 T0	290	45	0	11.8	Pos.	45	0	11.9	Pos.
13-VCF-STEPS-B-S08-300 Tf		33.2	0.1			33.1	0.1		
14-VCF-STEPS-B-S09-3,000 T0	2,900	45	0	13.9	Pos.	45	0	14.1	Pos.
14-VCF-STEPS-B-S09-3,000 Tf		31.1	0.1			30.9	0		
15-VCF-STEPS-B-S10-3,000 T0	2,900	45	0	17.8	Pos.	45	0	17.8	Pos.
15-VCF-STEPS-B-S10-3,000 Tf		27.2	0.1			27.2	0		
16-VCF-CARPET-A-S07-3,000 T0	2,900	45	0	20.1	Pos.	45	0	20	Pos.
16-VCF-CARPET-A-S07-3,000 Tf		24.9	0			25	0		
1-VCF-SCFILT-B-S04-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-VCF-SCFILT-B-S04-0 Tf		45	0			45	0		
2-VCF-SCFILT-B-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-VCF-SCFILT-B-S05-0 Tf		45	0			45	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
3-VCF-SCFILT-B-S06-30 T0	32	45	0	0	Neg.	45	0	0	Neg.
3-VCF-SCFILT-B-S06-30 Tf		45	0			45	0		
4-VCF-SCFILT-B-S07-30 T0	32	45	0	0	Neg.	45	0	0	Neg.
4-VCF-SCFILT-B-S07-30 Tf		45	0			45	0		
5-VCF-SCFILT-B-S08-300 T0	320	45	0	0	Neg.	45	0	9.8	Neg.
5-VCF-SCFILT-B-S08-300 Tf*		45	0			35.2	0.3		
6-VCF-SCFILT-B-S09-300 T0	320	45	0	0	Neg.	45	0	0	Neg.
6-VCF-SCFILT-B-S09-300 Tf		45	0			45	0		
7-VCF-SCFILT-B-S10-3,000 T0	3,200	45	0	2.7	Neg.	45	0	7.9	Neg.
7-VCF-SCFILT-B-S10-3,000 Tf*		42.3	4.8			37.1	0.5		
8-VCF-SCFILT-B-S11-3,000 T0	3,200	45	0	1.1	Neg.	45	0	0	Neg.
8-VCF-SCFILT-B-S11-3,000 Tf		43.9	1.9			45	0		
9-VCF-SWCON-A-S05-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-VCF-SWCON-A-S05-0 Tf		45	0			45	0		
10-VCF-SWCON-A-S06-30 T0	32	45	0	0	Neg.	45	0	0	Neg.
10-VCF-SWCON-A-S06-30 Tf		45	0			45	0		
11-VCF-SWCON-A-S07-30 T0	32	45	0	0	Neg.	45	0	0	Neg.
11-VCF-SWCON-A-S07-30 Tf		45	0			45	0		
12-VCF-SWCON-A-S08-300 T0	320	45	0	20.2	Pos.	45	0	20.1	Pos.
12-VCF-SWCON-A-S08-300 Tf		24.8	0.1			24.9	0		
13-VCF-SWCON-A-S09-300 T0	320	45	0	0	Neg.*	45	0	0	Neg.*
13-VCF-SWCON-A-S09-300 Tf		45	0			45	0		
14-VCF-SWCON-A-S10-3,000 T0	3,200	45	0	20.3	Pos.	45	0	20.2	Pos.
14-VCF-SWCON-A-S10-3,000 Tf		24.7	0.1			24.8	0		
15-VCF-SWCON-A-S11-3,000 T0	3,200	45	0	17	Pos.	45	0	17.3	Pos.
15-VCF-SWCON-A-S11-3,000 Tf		28	0			27.7	0.1		
16-VCF-LABBLK-3,000 T0	3,200	45	0	0	Neg.*	45	0	0	Neg.*
16-VCF-LABBLK-3,000 Tf		45	0			45	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
1-VCF-PAVEMT-A-S04-0 T0	0	45	0	7.2	Neg.	45	0	5.7	Neg.
1-VCF-PAVEMT-A-S04-0 Tf		37.8	0.1			39.3	2		
2-VCF-PAVEMT-A-S05-0 T0	0	45	0	5.1	Neg.	45	0	8	Neg.
2-VCF-PAVEMT-A-S05-0 Tf		39.9	4.4			37	0.1		
3-VCF-PAVEMT-A-S06-30 T0	26	45	0	7.9	Neg.	45	0	8.7	Neg.
3-VCF-PAVEMT-A-S06-30 Tf		37.1	0.9			36.3	0.5		
4-VCF-PAVEMT-A-S07-30 T0	26	45	0	13.8	Pos.	45	0	13.9	Pos.
4-VCF-PAVEMT-A-S07-30 Tf		31.2	0.1			31.1	0		
5-VCF-PAVEMT-A-S08-300 T0	260	45	0	8	Neg.	45	0	7.7	Neg.
5-VCF-PAVEMT-A-S08-300 Tf		37	0.8			37.3	0.5		
6-VCF-PAVEMT-A-S09-300 T0	260	45	0	2	Neg.	45	0	2.3	Neg.
6-VCF-PAVEMT-A-S09-300 Tf		43	3.5			42.7	3.9		
7-VCF-PAVEMT-A-S10-3,000 T0	2,600	45	0	20.5	Pos.	45	0	20.6	Pos.
7-VCF-PAVEMT-A-S10-3,000 Tf		24.5	0.1			24.4	0		
8-VCF-PAVEMT-A-S11-3,000 T0	2,600	45	0	20.7	Pos.	45	0	20.8	Pos.
8-VCF-PAVEMT-A-S11-3,000 Tf		24.3	0.1			24.2	0		
9-VCF-CARPET-A-S08-0 T0	0	45	0	6.7	Neg.	45	0	6.6	Neg.
9-VCF-CARPET-A-S08-0 Tf		38.3	0.6			38.4	1.3		
10-VCF-FLDBLK-A-S04-0 T0	0	45	0	7.7	Neg.	45	0	8.3	Neg.
10-VCF-FLDBLK-A-S04-0 Tf		37.3	1.2			36.7	0.5		
11-VCF-CARPET-A-S09-30 T0	26	45	0	10.9	Pos.	45	0	10.9	Pos.
11-VCF-CARPET-A-S09-30 Tf		34.1	0.2			34.1	0.2		
12-VCF-FLDBLK-A-S05-30 T0	26	45	0	5	Neg.	45	0	6.3	Neg.
12-VCF-FLDBLK-A-S05-30 Tf		40	4.3			38.7	1		
13-VCF-CARPET-A-S10-300 T0	260	45	0	18.4	Pos.	44	1.8	17.5	Pos.
13-VCF-CARPET-A-S10-300 Tf		26.6	0.1			26.5	0		
14-VCF-FLDBLK-A-S06-300 T0	260	45	0	0.6	Neg.	45	0	5.4	Neg.
14-VCF-FLDBLK-A-S06-300 Tf		44.4	1			39.6	0.6		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
15-VCF-CARPET-A-S11-3,000 T0	2,600	45	0	12	Pos.	45	0	12.1	Pos.
15-VCF-CARPET-A-S11-3,000 Tf		33	0.1			32.9	0.1		
16-VCF-FLDBLK-A-S07-3,000 T0	2,600	45	0	26.5	Pos.	44.6	0.8	26.1	Pos.
16-VCF-FLDBLK-A-S07-3,000 Tf		18.5	0			18.4	0		
1-VCF-FLCON-B-S11-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-VCF-FLCON-B-S11-0 Tf		45	0			45	0		
2-VCF-STEPS-B-S11-0 T0	0	45	0	0	Neg.	44.5	0.8	-0.5	Neg.
2-VCF-STEPS-B-S11-0 Tf		45	0			45	0		
3-VCF-FLCON-B-S12-30 T0	51	45	0	0	Neg.	45	0	0	Neg.
3-VCF-FLCON-B-S12-30 Tf		45	0			45	0		
4-VCF-STEPS-B-S12-30 T0	51	45	0	0	Neg.	45	0	1.7	Neg.
4-VCF-STEPS-B-S12-30 Tf		45	0			43.3	3		
5-VCF-FLCON-B-S13-300 T0	510	45	0	22.5	Pos.	45	0	23	Pos.
5-VCF-FLCON-B-S13-300 Tf		22.5	0			22	0		
6-VCF-STEPS-B-S13-300 T0	510	45	0	18.7	Pos.	45	0	18.4	Pos.
6-VCF-STEPS-B-S13-300 Tf		26.3	0			26.6	0		
7-VCF-FLCON-B-S14-3,000 T0	5,100	45	0	21.1	Pos.	45	0	21.4	Pos.
7-VCF-FLCON-B-S14-3,000 Tf		23.9	0			23.6	0		
8-VCF-STEPS-B-S14-3,000 T0	5,100	45	0	21.6	Pos.	45	0	21.7	Pos.
8-VCF-STEPS-B-S14-3,000 Tf		23.4	0.1			23.3	0.1		
9-VCF-CARPET-A-S12-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
9-VCF-CARPET-A-S12-0 Tf		45	0			45	0		
10-VCF-SCFILT-B-S12-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
10-VCF-SCFILT-B-S12-0 Tf		45	0			45	0		
11-VCF-CARPET-A-S13-30 T0	51	45	0	0	Neg.	45	0	0	Neg.
11-VCF-CARPET-A-S13-30 Tf		45	0			45	0		
12-VCF-SCFILT-B-S13-30 T0	51	45	0	0	Neg.	45	0	0.7	Neg.
12-VCF-SCFILT-B-S13-30 Tf		45	0			44.3	1.2		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
13-VCF-CARPET-A-S14-300 T0	510	45	0	17.9	Pos.	45	0	18.2	Pos.
13-VCF-CARPET-A-S14-300 Tf		27.1	0			26.8	0		
14-VCF-SCFILT-B-S14-300 T0	510	45	0	0	Neg.	45	0	0	Neg.
14-VCF-SCFILT-B-S14-300 Tf		45	0			45	0		
15-VCF-CARPET-A-S15-3,000 T0	5,100	45	0	21	Pos.	45	0	21.1	Pos.
15-VCF-CARPET-A-S15-3,000 Tf		24	0			23.9	0		
16-VCF-SCFILT-B-S15-3,000 T0	5,100	45	0	0	Neg.	44.4	1.1	-0.2	Neg.
16-VCF-SCFILT-B-S15-3,000 Tf		45	0			44.5	0.8		
1-VCF-FLDBLK-A-S08-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-VCF-FLDBLK-A-S08-0 Tf		45	0			45	0		
2-VCF-FLDBLK-A-S09-30 T0	30	45	0	0	Neg.	45	0	0	Neg.
2-VCF-FLDBLK-A-S09-30 Tf		45	0			45	0		
3-VCF-PAVEMT-A-S12-30 T0	30	45	0	0	Neg.	45	0	0	Neg.
3-VCF-PAVEMT-A-S12-30 Tf		45	0			45	0		
4-VCF-CARPET-A-S16-30 T0	30	45	0	0	Neg.	45	0	0	Neg.
4-VCF-CARPET-A-S16-30 Tf		45	0			45	0		
5-VCF-FLDBLK-A-S10-300 T0	300	45	0	0	Neg.	45	0	0	Neg.
5-VCF-FLDBLK-A-S10-300 Tf		45	0			45	0		
6-VCF-PAVEMT-A-S13-300 T0	300	45	0	7.4	Neg.	45	0	7.4	Neg.
6-VCF-PAVEMT-A-S13-300 Tf		37.6	0.3			37.6	0.5		
7-VCF-FLDBLK-A-S11-3,000 T0	3,000	45	0	0	Neg.	45	0	0	Neg.
7-VCF-FLDBLK-A-S11-3,000 Tf		45	0			45	0		
8-VCF-PAVEMT-A-S14-3,000 T0	3,000	45	0	14.1	Pos.	45	0	14.4	Pos.
8-VCF-PAVEMT-A-S14-3,000 Tf		30.9	0			30.6	0		
9-VCF-SCFILT-B-S16-30 T0	30	45	0	0	Neg.	45	0	0	Neg.
9-VCF-SCFILT-B-S16-30 Tf		45	0			45	0		
10-VCF-SWCON-A-S12-30 T0	30	45	0	2.4	Neg.	45	0	2.6	Neg.
10-VCF-SWCON-A-S12-30 Tf		42.6	4.2			42.4	2.5		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
11-VCF-CARPET-A-S17-300 T0	300	45	0	17.1	Pos.	45	0	16.9	Pos.
11-VCF-CARPET-A-S17-300 Tf		27.9	0.1			28.1	0		
12-VCF-SCFILT-B-S17-300 T0	300	45	0	0	Neg.	45	0	0	Neg.
12-VCF-SCFILT-B-S17-300 Tf		45	0			45	0		
13-VCF-SWCON-A-S13-300 T0	300	45	0	18.8	Pos.	45	0	18.8	Pos.
13-VCF-SWCON-A-S13-300 Tf		26.2	0.1			26.2	0		
14-VCF-CARPET-A-S18-3,000 T0	3,000	45	0	21.1	Pos.	45	0	21.1	Pos.
14-VCF-CARPET-A-S18-3,000 Tf		23.9	0			23.9	0		
15-VCF-SCFILT-B-S18-3,000 T0	3,000	45	0	0	Neg.	45	0	0	Neg.
15-VCF-SCFILT-B-S18-3,000 Tf		45	0			45	0		
16-VCF-SWCON-A-S14-3,000 T0	3,000	45	0	27.3	Pos.	45	0	27.6	Pos.
16-VCF-SWCON-A-S14-3,000 Tf		17.7	0			17.4	0		
1-VCF-FLCON-B-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
1-VCF-FLCON-B-S15-0 Tf		45	0			45	0		
2-VCF-STEPS-B-S15-0 T0	0	45	0	0	Neg.	45	0	0	Neg.
2-VCF-STEPS-B-S15-0 Tf		45	0			45	0		
3-VCF-FLCON-B-S16-30 T0	29	45	0	2	Neg.	45	0	2	Neg.
3-VCF-FLCON-B-S16-30 Tf		43	3.5			43	3.5		
4-VCF-STEPS-B-S16-30 T0	29	45	0	1.8	Neg.	45	0	0	Neg.
4-VCF-STEPS-B-S16-30 Tf		43.2	3.1			45	0		
5-VCF-FLCON-B-S17-300 T0	290	45	0	18.8	Pos.	45	0	18.6	Pos.
5-VCF-FLCON-B-S17-300 Tf		26.2	0			26.4	0		
6-VCF-STEPS-B-S17-300 T0	290	45	0	14.3	Pos.	45	0	14.8	Pos.
6-VCF-STEPS-B-S17-300 Tf		30.7	0.1			30.2	0.2		
7-VCF-FLCON-B-S18-3,000 T0	2,900	45	0	21.3	Pos.	45	0	21.2	Pos.
7-VCF-FLCON-B-S18-3,000 Tf		23.7	0.1			23.8	0		
8-VCF-STEPS-B-S18-3,000 T0	2,900	45	0	14	Pos.	45	0	14.1	Pos.
8-VCF-STEPS-B-S18-3,000 Tf		31	0.1			30.9	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
9-VCF-SWCON-A-S15-0 T0	0	45	0	0	Neg.	45	0	2.8	Neg.
9-VCF-SWCON-A-S15-0 Tf		45	0			42.2	2.5		
10-VCF-PAVEMT-A-S15-0 T0	0	45	0	2.3	Neg.	45	0	1.3	Neg.
10-VCF-PAVEMT-A-S15-0 Tf		42.7	4.1			43.7	2.2		
11-VCF-SWCON-A-S16-30 T0	29	45	0	0	Neg.	45	0	2.2	Neg.
11-VCF-SWCON-A-S16-30 Tf		45	0			42.8	2.1		
12-VCF-PAVEMT-A-S16-30 T0	29	45	0	0	Neg.	45	0	3.7	Neg.
12-VCF-PAVEMT-A-S16-30 Tf		45	0			41.3	3.3		
13-VCF-SWCON-A-S17-300 T0	290	45	0	22.9	Pos.	45	0	23.1	Pos.
13-VCF-SWCON-A-S17-300 Tf		22.1	0.1			21.9	0		
14-VCF-PAVEMT-A-S17-300 T0	290	45	0	4	Neg.	45	0	7.8	Neg.
14-VCF-PAVEMT-A-S17-300 Tf		41	3.5			37.2	0.3		
15-VCF-SWCON-A-S18-3,000 T0	2,900	45	0	29.1	Pos.	45	0	29.3	Pos.
15-VCF-SWCON-A-S18-3,000 Tf		15.9	0			15.7	0		
16-VCF-PAVEMT-A-S18-3,000 T0	2,900	45	0	10.6	Pos.	45	0	11.1	Pos.
16-VCF-PAVEMT-A-S18-3,000 Tf		34.4	0.3			33.9	0.2		
1-VCF-FLDBLK-A-S12-0 T0	0	45	0	2.4	Neg.	45	0	4.4	Neg.
1-VCF-FLDBLK-A-S12-0 Tf		42.6	4.2			40.6	3.8		
2-VCF-FLDBLK-A-S13-30 T0	19	45	0	0	Neg.	45	0	0	Neg.
2-VCF-FLDBLK-A-S13-30 Tf		45	0			45	0		
3-VCF-FLDBLK-A-S14-30 T0	19	45	0	0	Neg.	41.8	2.8	-1.5	Neg.
3-VCF-FLDBLK-A-S14-30 Tf		45	0			43.3	2.9		
4-VCF-FLDBLK-A-S15-300 T0	190	45	0	0	Neg.	45	0	0	Neg.
4-VCF-FLDBLK-A-S15-300 Tf		45	0			45	0		
5-VCF-FLDBLK-A-S16-300 T0	190	45	0	0	Neg.	45	0	0	Neg.
5-VCF-FLDBLK-A-S16-300 Tf		45	0			45	0		
6-VCF-FLDBLK-A-S17-3000 T0	1,900	45	0	24.2	Pos.	45	0	24.1	Pos.
6-VCF-FLDBLK-A-S17-3000 Tf		20.8	0			20.9	0		

Sample ID	Spore Load	Chrom. Average Ct	Chrom. Stnd Dev	Chom. ΔCt	Chrom. Result	pXO1 Average Ct	pXO1 Stnd Dev	pXO1 Chom. ΔCt	pXO1 Chrom. Result
7-VCF-FLDBLK-A-S18-3000 T0	1,900	45	0	24.1	Pos.	43.3	3	22.3	Pos.
7-VCF-FLDBLK-A-S18-3000 Tf		20.9	0			21	0		

APPENDIX S: TSB ENRICHMENT PCR RESULTS FOR SPONGE-STICK SAMPLES

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
2-SPG-STGRAT-A-S15-0*	45.0	0.0	Neg	41.4	3.1	Pos	22
4-SPG-STGRAT-A-S16-30*	38.6	5.6	Pos	38.4	5.8	Pos	22
6-SPG-STGRAT-A-S17-300*	40.3	4.1	Pos	38.4	1.1	Pos	22
8-SPG-STGRAT-A-S18-3000*	36.8	0.8	Pos	36.5	0.7	Pos	22
10-SPG-CWPNTD-A-S16-30	45.0	0.0	Neg	45.0	0.0	Neg	21
1-SPG-FLDBLK-A-S15-0*	44.7	0.5	Neg	38.5	0.4	Pos	22
2-SPG-MCMACH-B-S15-0	45.0	0.0	Neg	45.0	0.0	Neg	21
4-SPG-MCMACH-B-S16-30	45.0	0.0	Neg	45.0	0.0	Neg	21
1-SPG-WLTILE-B-S15-0	45.0	0.0	Neg	45.0	0.0	Neg	21
10-SPG-WLTILE-B-S19-0	45.5	0.0	Neg	44.6	0.7	Neg	22
9-SPG-SCGRIL-B-S13-0	45.0	0.0	Neg	45.0	0.0	Neg	20
13-SPG-SCGRIL-B-S15-30	45.0	0.0	Neg	45.0	0.0	Neg	20
2-SPG-STEPS-B-S15-0	45.0	0.0	Neg	45.0	0.0	Neg	20
6-SPG-STEPS-B-S17-300	45.0	0.0	Neg	45.0	0.0	Neg	20
1-SPG-FLCON-B-S15-0	45.0	0.0	Neg	45.0	0.0	Neg	20
3-SPG-FLCON-B-S16-30	45.0	0.0	Neg	45.0	0.0	Neg	20
10-SPG-LABBLANK-0	45.0	0.0	Neg	45.0	0.0	Neg	20
9-SPG-GRNBEN-A-S15-0	45.0	0.0	Neg	40.8	3.7	Pos	22
9-SPG-TELEBO-A-S12-0	45.0	0.0	Neg	45.0	0.0	Neg	19
10-SPG-TELEBO-A-S13-30	45.0	0.0	Neg	45.0	0.0	Neg	19
1-SPG-CWSIGN-A-S13-0	45.0	0.0	Neg	45.0	0.0	Neg	19

*Note, these enrichment samples PCR analyzed the week of December 3, 2018. The Tfinal 0 spike samples also had Ct in of ~ 37, therefore contamination may have caused these positive results.

APPENDIX T: TSB ENRICHMENT PCR RESULTS FOR VACUUM FILTER CASSETTES

Concrete Floor VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
1-VCF-FLCON-B-S01-0	45.0	0.0	Neg.	45.0	0.0	Neg.	2
1-VCF-FLCON-B-S11-0	45.0	0.0	Neg.	44.1	1.5	Neg.	6
1-VCF-FLCON-B-S15-0	40.3	4.1	Pos.	38.1	0.8	Pos.	8
2-VCF-FLCON-B-S02-30	32.5	1.1	Pos.	32.5	0.9	Pos.	2
1-VCF-FLCON-B-S05-30	35.4	0.5	Pos.	34.7	0.4	Pos.	3
2-VCF-FLCON-B-S06-30	32.4	0.5	Pos.	32.3	0.5	Pos.	3
3-VCF-FLCON-B-S12-30	38.6	5.5	Pos.	37.8	5.1	Pos.	6
3-VCF-FLCON-B-S16-30	31.7	0.1	Pos.	31.2	0.1	Pos.	8
5-VCF-FLCON-B-S03-300	30.2	0.2	Pos.	29.6	0.1	Pos.	2
5-VCF-FLCON-B-S07-300	29.3	0.3	Pos.	29.2	0.3	Pos.	3
6-VCF-FLCON-B-S08-300	32.1	0.4	Pos.	31.8	0.4	Pos.	3
5-VCF-FLCON-B-S13-300	29.4	0.2	Pos.	29.2	0.2	Pos.	6
5-VCF-FLCON-B-S17-300	30.5	0.1	Pos.	30.2	0	Pos.	8
7-VCF-FLCON-B-S04-3,000	29.1	0.2	Pos.	29.1	0.2	Pos.	2
7-VCF-FLCON-B-S09-3,000	28.2	0.5	Pos.	27.8	0.5	Pos.	3
8-VCF-FLCON-B-S10-3,000	25.6	0.3	Pos.	25.4	0.3	Pos.	3
7-VCF-FLCON-B-S14-3,000	28.1	0.0	Pos.	27.8	0.1	Pos.	6
7-VCF-FLCON-B-S18-3,000	27.1	0.1	Pos.	26.8	0	Pos.	8

Zero spike sample positive. One of three replicates had Ct = 45, other two replicates were Ct of 37.

Step VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
2-VCF-STEPS-B-S11-0	45.0	0.0	Neg.	45.0	0.0	Neg.	6
2-VCF-STEPS-B-S15-0	45.0	0.0	Neg.	45.0	0.0	Neg.	8
3-VCF-STEPS-B-S05-30	45.0	0.0	Neg.	43.3	3.0	Neg.	3
4-VCF-STEPS-B-S06-30	45.0	0.0	Neg.	43.3	3.0	Neg.	3
4-VCF-STEPS-B-S12-30	43.1	3.2	Neg.	45.0	0.0	Neg.	6
4-VCF-STEPS-B-S16-30	45.0	0.0	Neg.	43.4	2.7	Neg.	8
12-VCF-STEPS-B-S07-300	33.9	0.2	Pos.	33.1	0.2	Pos.	3
13-VCF-STEPS-B-S08-300	42.3	4.0	Neg.	38.7	1.2	Pos.	3
6-VCF-STEPS-B-S13-300	38.6	5.5	Pos.	38.6	5.5	Pos.	6
6-VCF-STEPS-B-S17-300	40.0	4.4	Pos.	39.0	0.9	Pos.	8
14-VCF-STEPS-B-S09-3,000	35.2	0.7	Pos.	35.0	0.4	Pos.	3
15-VCF-STEPS-B-S10-3,000	36.2	0.2	Pos.	35.1	0.1	Pos.	3
8-VCF-STEPS-B-S14-3,000	38.4	5.7	Pos.	38.4	5.7	Pos.	6
8-VCF-STEPS-B-S18-3,000	34.8	0.2	Pos.	34.5	0.1	Pos.	8

Carpet VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
9-VCF-CARPET-A-S04-0	45.0	0.0	Neg.	45.0	0.0	Neg.	3
9-VCF-CARPET-A-S08-0	45.0	0.0	Neg.	45.0	0.0	Neg.	5
9-VCF-CARPET-A-S12-0	45.0	0.0	Neg.	45.0	0.0	Neg.	6
3-VCF-CARPET-A-S01-30	36.6	0.7	Pos.	35.6	0.5	Pos.	2
10-VCF-CARPET-A-S05-30	36.2	0.6	Pos.	36.2	0.8	Pos.	3
11-VCF-CARPET-A-S09-30	45.0	0.0	Neg.	40.4	4.0	Pos.	5
11-VCF-CARPET-A-S13-30	41.0	3.7	Pos.	37.7	0.2	Pos.	6
4-VCF-CARPET-A-S16-30	38	0.8	Pos.	38.4	0.8	Pos.	7
6-VCF-CARPET-A-S02-300	42.9	3.7	Neg.	40.0	4.3	Pos.	2
11-VCF-CARPET-A-S06-300	34.7	0.2	Pos.	34.1	0.4	Pos.	3
13-VCF-CARPET-A-S10-300	32.8	0.0	Pos.	32.6	0.1	Pos.	5
13-VCF-CARPET-A-S14-300	37.0	1.2	Pos.	35.6	0.6	Pos.	6
11-VCF-CARPET-A-S17-300	36.4	0.6	Pos.	35.8	0.1	Pos.	7
8-VCF-CARPET-A-S03-3,000	34.9	0.1	Pos.	34.1	0.3	Pos.	2
16-VCF-CARPET-A-S07-3,000	31.4	0.3	Pos.	30.8	0.1	Pos.	3
15-VCF-CARPET-A-S11-3,000	32.1	0.1	Pos.	31.7	0.1	Pos.	5
15-VCF-CARPET-A-S15-3,000	32.9	0.1	Pos.	32.7	0.1	Pos.	6
14-VCF-CARPET-A-S18-3,000	32.1	0.1	Pos.	31.9	0.1	Pos.	7

Subway Car Filter VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
1-VCF-SCFILT-B-S04-0	41.5	3.0	Pos.	35.4	0.3	Pos.	4
2-VCF-SCFILT-B-S05-0	45.0	0.0	Neg.	45.0	0.0	Neg.	4
10-VCF-SCFILT-B-S12-0	45.0	0.0	Neg.	45.0	0.0	Neg.	6
4-VCF-SCFILT-B-S01-30	45.0	0.0	Neg.	45.0	0.0	Neg.	2
3-VCF-SCFILT-B-S06-30	42.7	3.9	Neg.	33.7	0.2	Pos.	4
4-VCF-SCFILT-B-S07-30	42.3	2.5	Neg.	35.7	0.4	Pos.	4
12-VCF-SCFILT-B-S13-30	44.1	1.5	Neg.	41.1	3.4	Pos.	6
9-VCF-SCFILT-B-S16-30	45	0	Neg.	45	0	Neg.	7
11-VCF-SCFILT-B-S02-300	36.9	0.8	Pos.	36.3	0.4	Pos.	2
5-VCF-SCFILT-B-S08-300	45.0	0.0	Neg.	45.0	0.0	Neg.	4
6-VCF-SCFILT-B-S09-300	45.0	0.0	Neg.	44.0	1.8	Neg.	4
14-VCF-SCFILT-B-S14-300	45.0	0.0	Neg.	45.0	0.0	Neg.	6
12-VCF-SCFILT-B-S17-300	41	3.5	Pos.	40.7	1.6	Neg.	7
14-VCF-SCFILT-B-S03-3000	35.2	0.3	Pos.	34.7	0.1	Pos.	2
7-VCF-SCFILT-B-S10-3,000	45.0	0.0	Neg.	45.0	0.0	Neg.	4
8-VCF-SCFILT-B-S11-3,000	43.9	1.8	Neg.	39.5	4.8	Pos.	4
16-VCF-SCFILT-B-S15-3,000	41.2	3.4	Pos.	40.8	3.7	Pos.	6
15-VCF-SCFILT-B-S18-3,000	42.4	4.4	Neg.	36.8	0.3	Pos.	7

Sidewalk Concrete VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
9-VCF-SWCON-A-S05-0	45.0	0.0	Neg.	45.0	0.0	Neg.	4
9-VCF-SWCON-A-S15-0	45.0	0.0	Neg.	45.0	0.0	Neg.	8
10-VCF-SWCON-A-S06-30	36.4	1.1	Pos.	35.9	0.7	Pos.	4
11-VCF-SWCON-A-S07-30	36.5	1.6	Pos.	36.2	0.5	Pos.	4
10-VCF-SWCON-A-S12-30	35.0	2.3	Pos.	35.6	2.7	Pos.	7
11-VCF-SWCON-A-S16-30	36.1	0.7	Pos.	36.1	0.2	Pos.	8
12-VCF-SWCON-A-S08-300	32.7	0.4	Pos.	32.5	0.4	Pos.	4
13-VCF-SWCON-A-S09-300	29.2	0.9	Pos.	28.9	0.9	Pos.	4
13-VCF-SWCON-A-S13-300	31.2	0.1	Pos.	30.8	0.1	Pos.	7
13-VCF-SWCON-A-S17-300	33.0	1.2	Pos.	33.1	1.4	Pos.	8
14-VCF-SWCON-A-S10-3,000 [(a)]	26.2	0.1	Pos.	25.9	0.1	Pos.	4
15-VCF-SWCON-A-S11-3,000	30.0	0.2	Pos.	30.0	0.2	Pos.	4
16-VCF-SWCON-A-S14-3,000	29.4	0.1	Pos.	29.7	0.1	Pos.	7
15-VCF-SWCON-A-S18-3,000	28.6	0.3	Pos.	28.7	0.3	Pos.	8

(a)Sample 14 was analyzed held at 2 - 8 C for 1 week before being analyzed

Pavement VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Stnd Dev	Result	Average Ct	Stnd Dev	Result	
1-VCF-PAVEMT-A-S04-0	42.5	4.4	Neg.	45.0	0.0	Neg.	5
2-VCF-PAVEMT-A-S05-0	45.0	0.0	Neg.	43.0	3.5	Neg.	5
10-VCF-PAVEMT-A-S15-0	45	0	Neg.	45	0	Neg.	8
9-VCF-PAVEMT-A-S01-30	36.8	0.3	Pos.	36.0	0.2	Pos.	2
3-VCF-PAVEMT-A-S06-30	43.0	3.5	Neg.	42.7	2.9	Neg.	5
4-VCF-PAVEMT-A-S07-30	35.6	0.3	Pos.	35.0	0.2	Pos.	5
3-VCF-PAVEMT-A-S12-30	39	0.5	Pos.	39.2	0.6	Pos.	7
12-VCF-PAVEMT-A-S16-30	33	0.2	Pos.	32.9	0	Pos.	8
12-VCF-PAVEMT-A-S02-300	32.1	0.7	Pos.	32.0	0.7	Pos.	2
5-VCF-PAVEMT-A-S08-300	33.4	0.4	Pos.	32.7	0.4	Pos.	5
6-VCF-PAVEMT-A-S09-300	36.9	1.5	Pos.	36.6	0.3	Pos.	5
6-VCF-PAVEMT-A-S13-300	33.7	0.6	Pos.	33.8	0.4	Pos.	7
14-VCF-PAVEMT-A-S17-300	30.6	0.4	Pos.	30.8	0.4	Pos.	8
15-VCF-PAVEMT-A-S03-3000	30.9	0.4	Pos.	31.0	0.4	Pos.	2
7-VCF-PAVEMT-A-S10-3,000	30.7	0.1	Pos.	30.5	0.0	Pos.	5
8-VCF-PAVEMT-A-S11-3,000	29.5	0.2	Pos.	29.1	0.2	Pos.	5
8-VCF-PAVEMT-A-S14-3,000	29.3	0.1	Pos.	29	0	Pos.	7
16-VCF-PAVEMT-A-S18-3,000	28.2	0.1	Pos.	28.1	0.2	Pos.	8

Field Blank VCF Samples

Sample ID	Chromosome Assay			pXO1 Assay			Trial
	Average Ct	Std Dev	Result	Average Ct	Std Dev	Result	
10-VCF-FLDBLK-A-S04-0	45.0	0.0	Neg.	44.8	0.4	Neg.	5
1-VCF-FLDBLK-A-S08-0	45.0	0.0	Neg.	45.0	0.0	Neg.	7
1-VCF-FLDBLK-A-S12-0	45.0	0.0	Neg.	45.0	0.0	Neg.	9
12-VCF-FLDBLK-A-S05-30	22.4	0.8	Pos.	22.4	1.0	Pos.	5
10-VCF-FLDBLK-A-S01-30	24.2	0.1	Pos.	24.2	0.1	Pos.	2
2-VCF-FLDBLK-A-S09-30	25.7	0.4	Pos.	25.6	0.4	Pos.	7
2-VCF-FLDBLK-A-S13-30	27.9	0.0	Pos.	28.1	0.0	Pos.	9
3-VCF-FLDBLK-A-S14-30	26.5	0.1	Pos.	26.8	0.1	Pos.	9
5-VCF-FLDBLK-A-S10-300	21.3	0.4	Pos.	21.3	0.5	Pos.	7
13-VCF-FLDBLK-A-S02-300	20.1	0.1	Pos.	20.1	0.1	Pos.	2
14-VCF-FLDBLK-A-S06-300	25.2	0.4	Pos.	25.4	0.5	Pos.	5
4-VCF-FLDBLK-A-S15-300	26.1	0.3	Pos.	26.2	0.3	Pos.	9
5-VCF-FLDBLK-A-S16-300	26.1	0.2	Pos.	26.3	0.4	Pos.	9
16-VCF-FLDBLK-A-S03-3000	21.1	1.3	Pos.	21.1	1.4	Pos.	2
16-VCF-FLDBLK-A-S07-3,000	21.3	0.1	Pos.	21.4	0.1	Pos.	5
7-VCF-FLDBLK-A-S11-3,000	21.9	0.2	Pos.	21.9	0.3	Pos.	7
6-VCF-FLDBLK-A-S17-3000	26.1	0.1	Pos.	26.3	0.1	Pos.	9
7-VCF-FLDBLK-A-S18-3000	25.6	0.1	Pos.	25.8	0.2	Pos.	9

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