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

- The U.S. Environmental Protection Agency (EPA) through its Office of Research and Development (ORD) funded and managed the research described. It has been subjected to the Agency's review and has been approved for publication and distribution. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Mention of trade names, products, or services does not convey official EPA approval, endorsement, or recommendation.
- Battelle is a contractor to EPA and provided technical support for the work described.
- Dr. Worth Calfee (EPA) was the Principal Investigator for this effort

Background

- EPA is responsible for remediation of land and public infrastructure following biological contamination involving *Bacillus anthracis*
  - *Emergency Support Function #10 of National Response Framework*
- Following a biological contamination incident, spatial extent of contamination should be determined using established sampling and analytical methods
- EPA and CDC have developed analytical methods and established sampling methods for Sponge-Sticks and vacuum filter cassettes (VFC)
- Collected and recovered real-world interferents (RWIs) may adversely impact quantification and identification of *B. anthracis* spores

Objective

- Assess the impact of RWIs collected on Sponge-Stick and VFC samples on the current EPA-developed culture and molecular methods for quantification and identification of viable *B. anthracis* spores in environmental samples

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Technical Approach - Overview

- Sampling campaign conducted in mid-town Manhattan (November 2017)
  - Times Square
  - Grand Central Station
- End-to-end assessment of *B. anthracis* spore recovery and detection from Sponge-Stick and VFC in the presence of RWIs

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Technical Approach – Sponge-Sticks

3M Sponge-Stick

Electronic Display Panel
(Times Square)

Surfaces Sampled	
Floor (Tile)	Metro Card Machine
Floor (Concrete)	Subway Car Filter Grille
Steps (w/Metal Grid)	Sidewalk Concrete
Wall Tile	Electrical Display Panel
Glass Window	Crosswalk Signal
Electrical Display Panel	Telephone Booth
Glass Panel	Street Grating
Fluor Light Fixture	Crosswalk Painted
Overhead Sign	Granite Bench

- Eighteen surfaces, plus field blank
- Target Spore Loads of 0/30/300/3,000
- Replicates of 3/5/5/5

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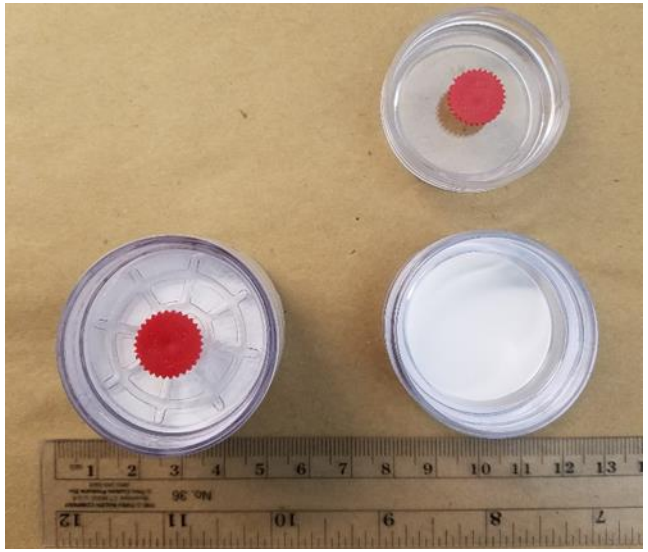
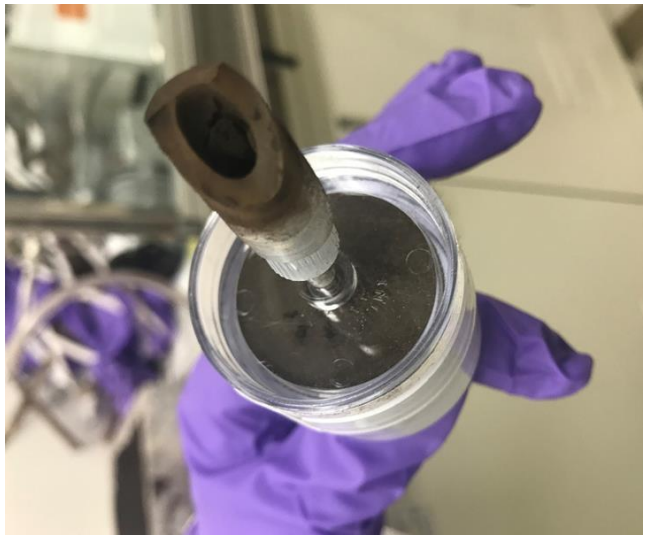
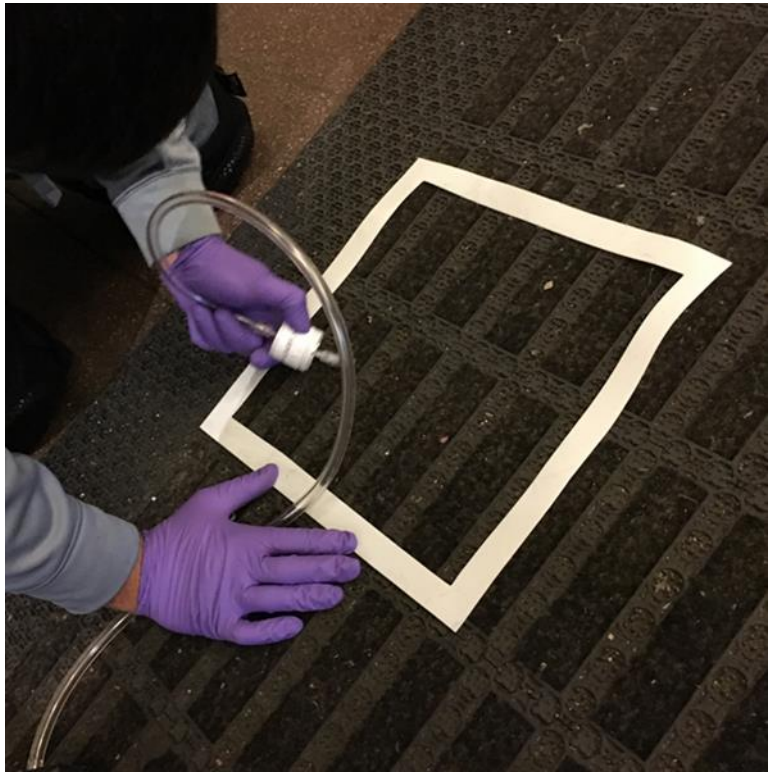
Technical Approach – Vacuum Filter Cassette (VFC)





Vacuum Filter Cassette

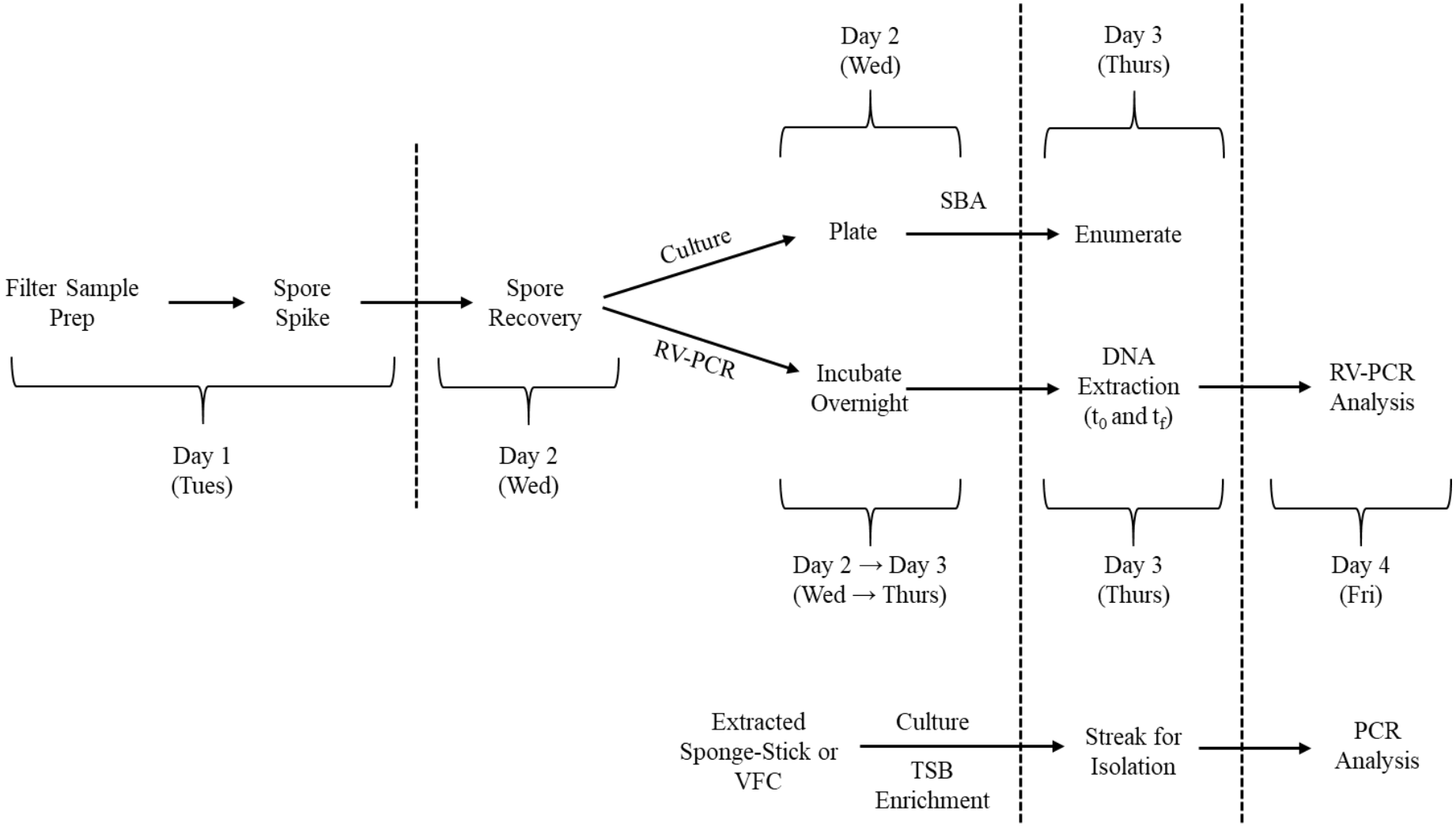


Carpet at Subway Station

Surfaces Sampled	
Floor (Concrete)	Subway Car HVAC Filter
Steps (w/Metal Grid)	Carpet/Rug
Sidewalk Concrete	Pavement (Asphalt)

- Six surfaces, plus field blank
  - Floor, Steps and Sidewalk also sampled via Sponge-Sticks
- Target Spore Loads of 0/30/300/3,000
- Replicates of 3/5/5/5

Technical Approach – Process Flow



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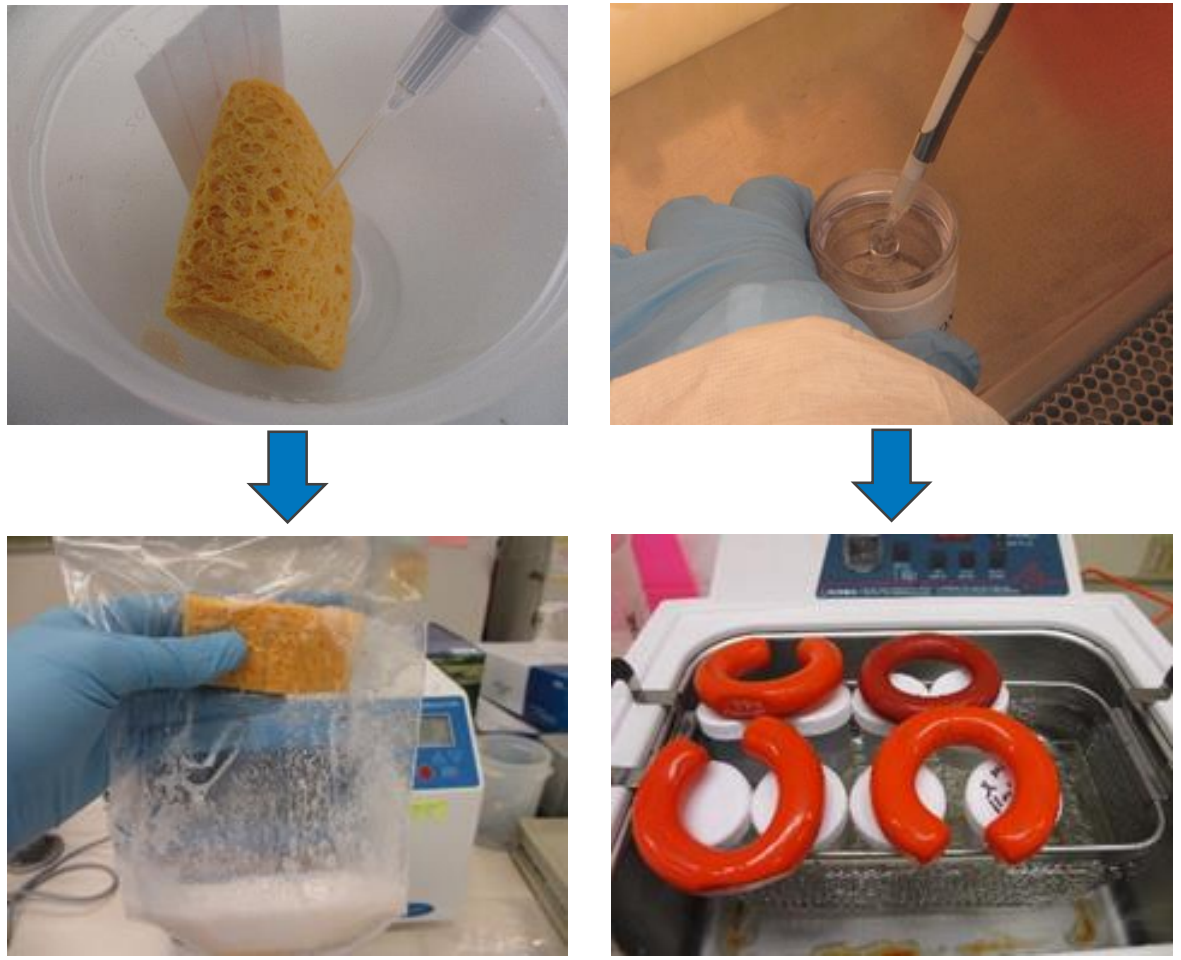
Technical Approach – Spore Spiking; Spore Recovery; Split Sample

- Spore Spiking (post-interferent collection)
  - B. a. Sterne spores
  - Pipetted twenty (20) 5 µL droplets
- Spore Recovery
  - Stomacher or sonication with cold extraction buffers
- Split recovered spores
  - Culture
  - Rapid Viability-PCR (RV-PCR)



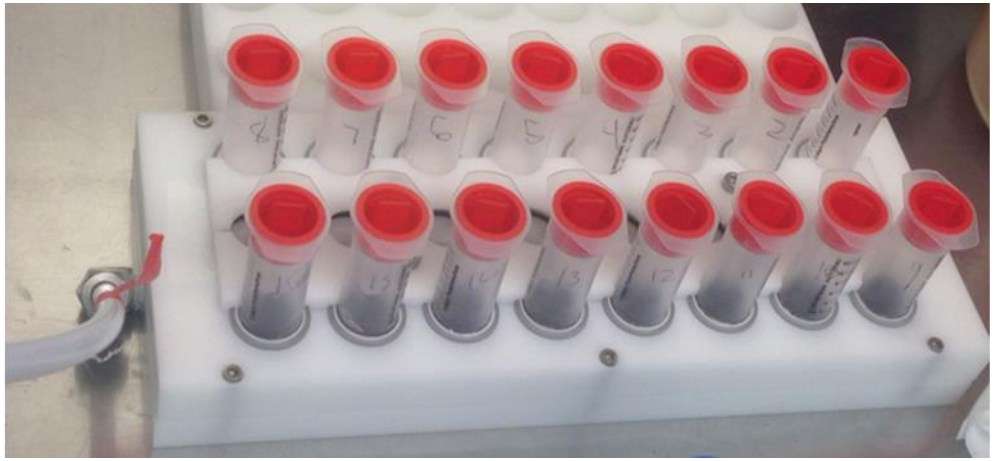
Technical Approach – Analytical Methods

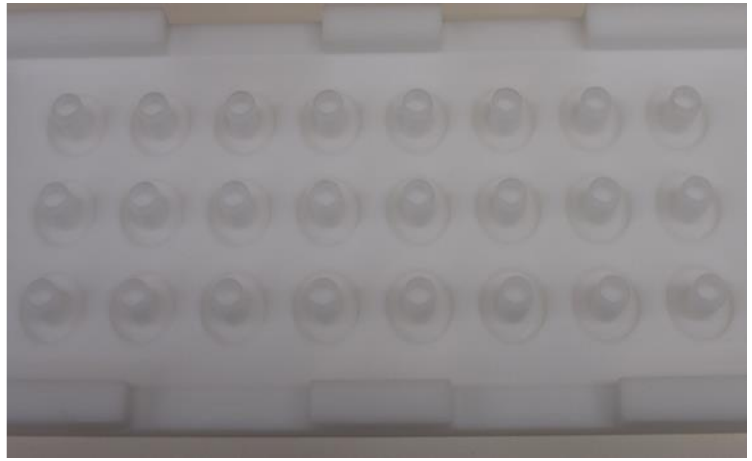
Culture

- Trypticase Soy Agar with 5% Sheep Blood (SBA)
- Colony PCR confirmation
- Trypticase Soy Broth enrichment

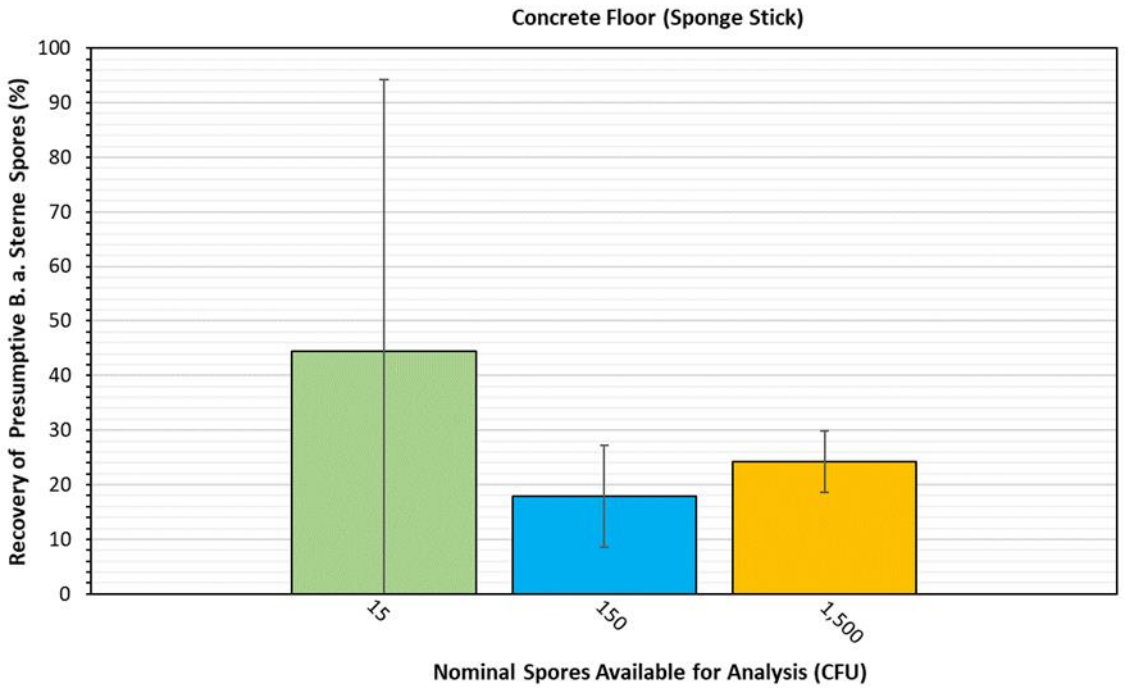
RV-PCR

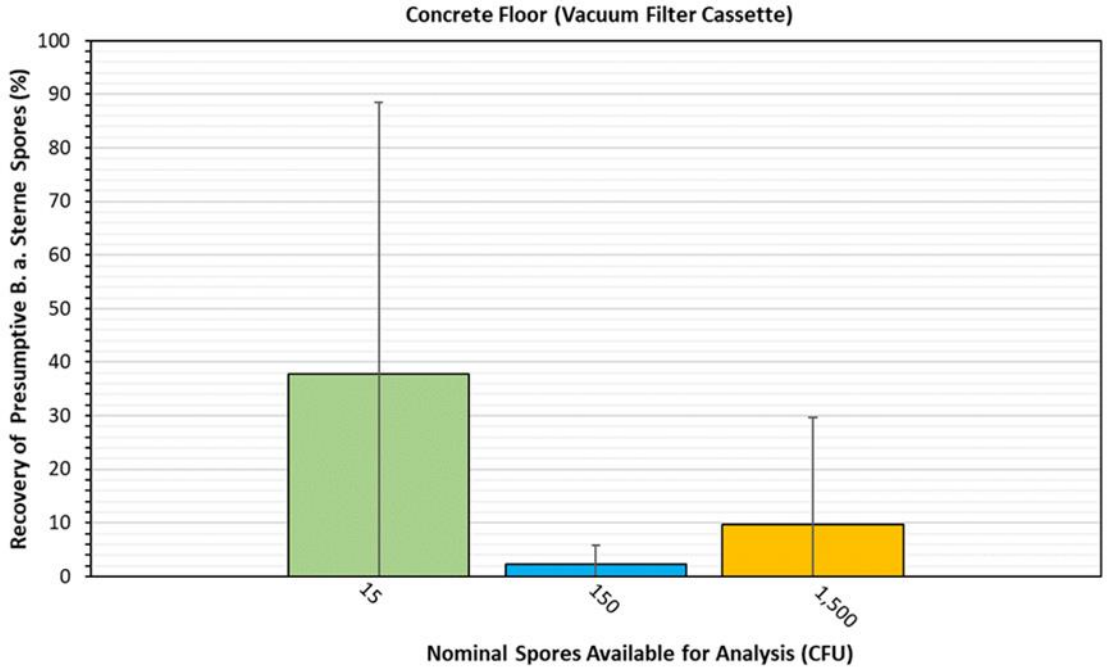
- Extract DNA from T_0 and T_f aliquots
- Real-Time PCR
  - Chromosome and pXO1 targets
- ΔCt values reported
  - ΔCt ≥9 reported as positive result





Culture Results – Representative Recovery Efficiencies





- Higher standard deviation for nominal 15 spores available attributed to relatively few (<10) recovered colony forming units (CFU)
- Lower percent recovery for VFCs attributed to spores being retained on the MCE filter substrate
- Application of spore using droplets may have adverse impact on recovery

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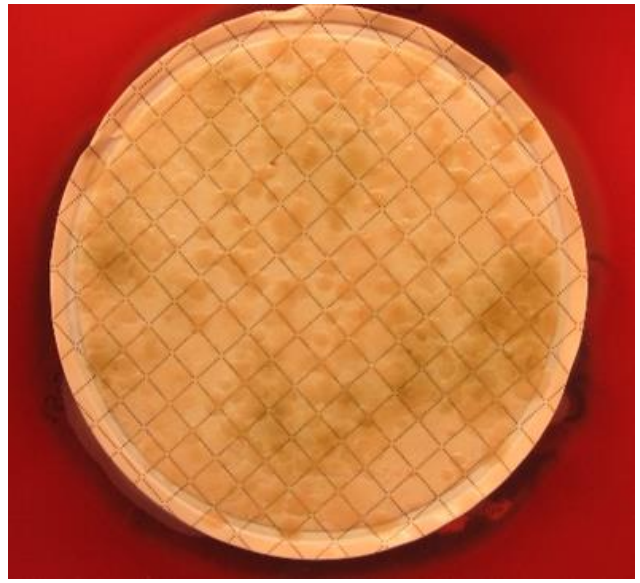
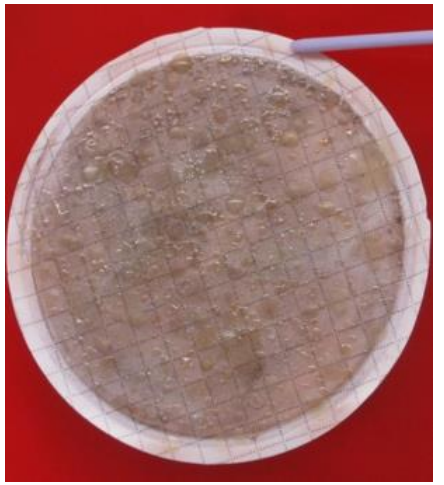
Culture Results – Background Flora/Grime Adversely Affected B. a. Sterne Quantification for Sponge-Stick

- A subset of colonies recovered were screened using real-time PCR assays targeting chromosomal and pXO1 gene targets
  - Of 229 colonies screened from Sponge-Sticks, 93% were confirmed as correctly identified
- Overall, background flora interfered with identification of presumptive B. a. Sterne from Street Grate samples to a greater degree than the other surfaces
  - All Street Grate samples had background flora counts of greater than 83 colonies
- 3 of 21 Sponge-Sticks that were TSB enriched were real-time PCR positive
  - Isolated colonies from turbid broth were all negative despite B. a. Sterne morphology on SBA

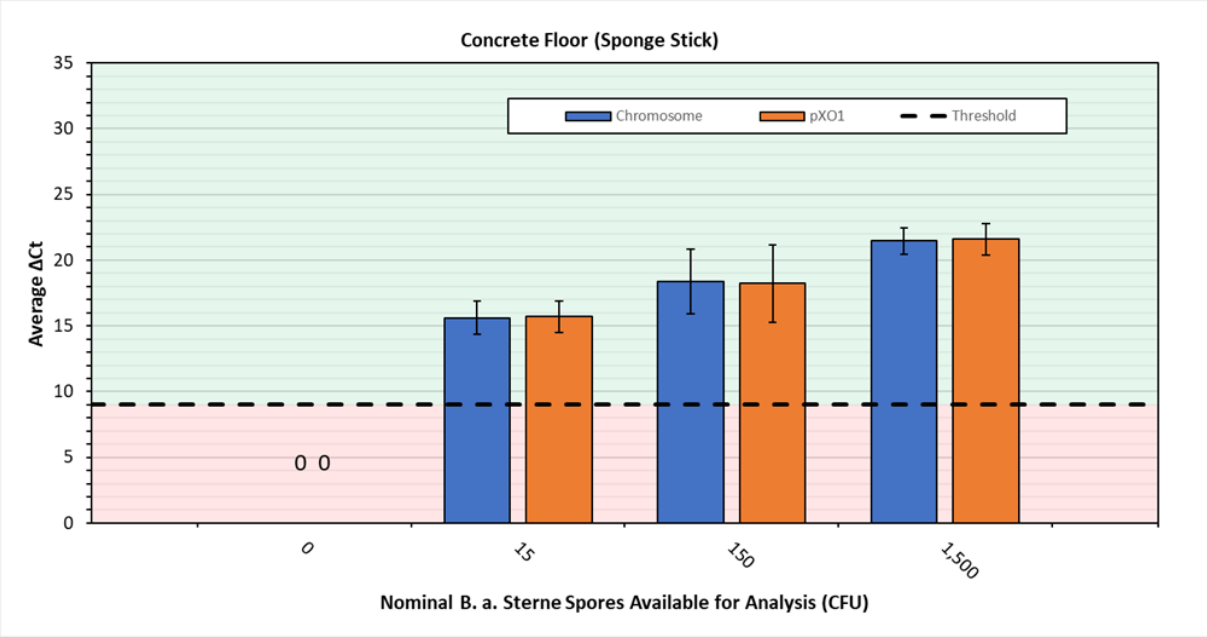


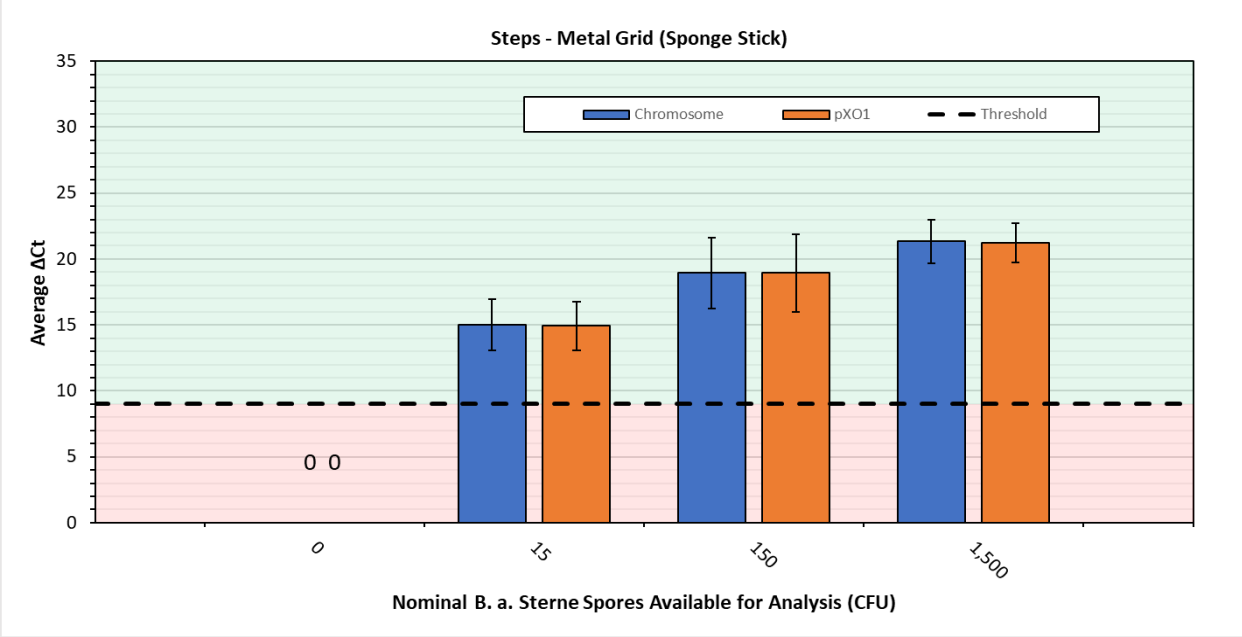
Culture Results – Background Flora/Grime Adversely Affected B. a. Sterne Quantification for VFC

- A subset of colonies recovered were screened using real-time PCR assays targeting chromosomal and pXO1 gene targets
  - Of 50 colonies screened from VFC, 68% were confirmed as correctly identified
  - 16 presumptive B. a. Sterne colonies that were real-time PCR negative artificially inflated the percent recovery
- Subway Car Filters appeared (visually) to be the dirtiest of the VFC filters
- TSB broth enrichment was PCR positive at a lower spore loading level than RV-PCR positive, indicating spores are not being physically removed from the filter of VFC samples
  - B. a. Sterne morphology was not isolated when turbid TSB broth was streaked onto SBA



RV-PCR Results – Representative ΔCt Values for Sponge-Sticks





- All RV-PCR Sponge-Sticks with a nominal 15 B. a. Sterne spores (CFU) available were positive except Street Grate and Painted Crosswalk
- Both chromosome and pXO1 gene targets in the RV-PCR assay yield comparable response

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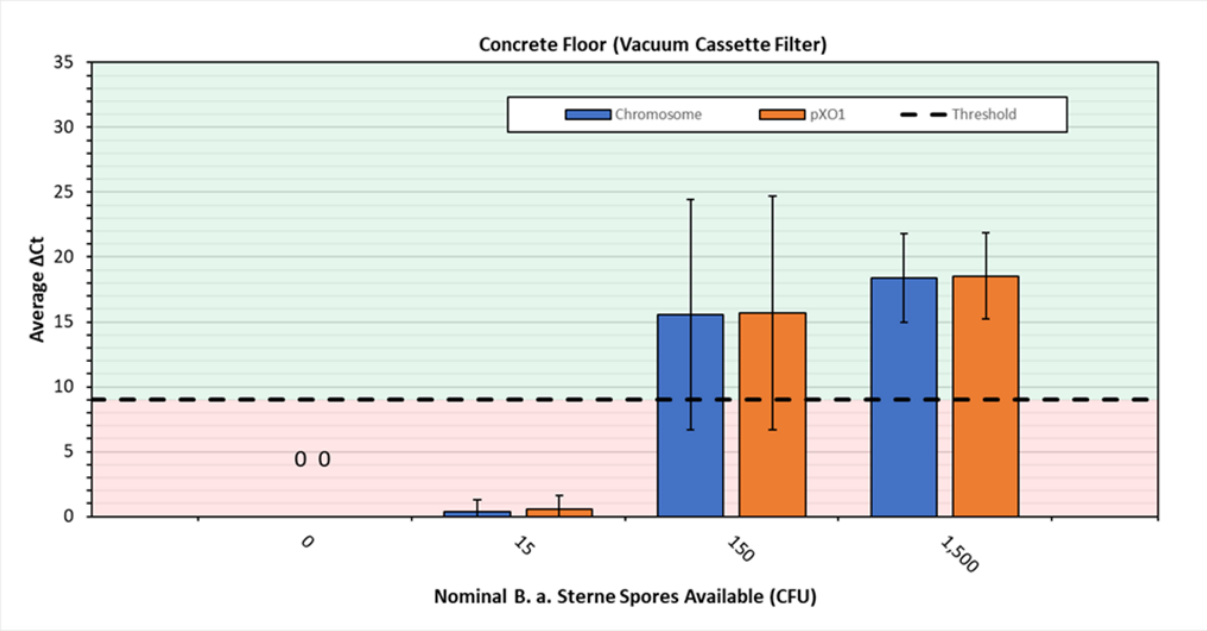
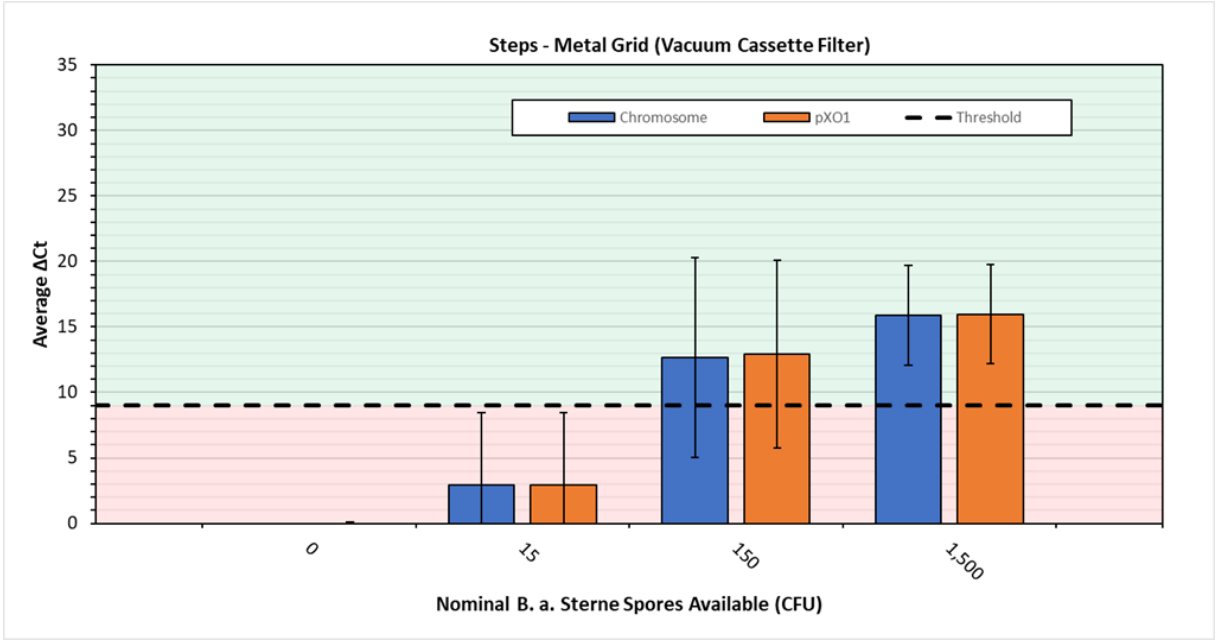
RV-PCR Results – Representative ΔCt Values for VFC





- All RV-PCR VFCs with a nominal 15 B. a. Sterne spores (CFU) available were negative
- Poor recovery efficiencies (< 10%) as determined by culture contributed to the low accuracy of RV-PCR detects
  - Field blank samples were non-detects for samples with 0, 15, and 150 B. a. Sterne spores nominally available

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Summary of Detection Accuracy – Sponge Sticks

Culture (SBA)				Molecular Response (RV-PCR)			
True Positive	**True Negative**	**False Positive**	**False Negative**	**True Positive**	**True Negative**	**False Positive**	**False Negative**
77% (220 of 285)	96% (55 of 57)	3.4% (2 of 57)	23% (65 of 285)	97% (276 of 285)	100% (57 of 57)	0% (0 of 57)	3.2% (9 of 285)

- Culture results
  - Two false positives, one each from Telephone Booth and Sidewalk Concrete
  - Surfaces with most false negatives: Street Grate (15), Crosswalk Painted (10), and Steps (8)
- RV-PCR results
  - Zero false positives
  - Two surfaces with more than 1 false negative: Street Grate (3) and Crosswalk Painted (2)

Summary of Detection Accuracy – VFCs

Culture (SBA)				Molecular Response (RV-PCR)			
True Positive	**True Negative**	**False Positive**	**False Negative**	**True Positive**	**True Negative**	**False Positive**	**False Negative**
54% (57 of 105)	90% (19 of 21)	10% (2 of 21)	46% (48 of 105)	47% (49 of 105)	100% (21 of 21)	0% (0 of 21)	52% (55 of 105

- Culture results
  - Two false positives, one each from Floor Concrete and Carpet
  - Surfaces with most false negatives: Subway Car Filter (8), Field Blank (8), and Carpet (8)
- RV-PCR results
  - Zero false positives
  - Surfaces with most false negatives: Subway Car Filter (14), Field Blank (11), and Pavement (10)

Summary of Key Findings

- *B. anthracis* analysis methods were 77% (Culture) and 97% (RV-PCR) accurate in correctly identifying the presence of B. a. Sterne in Sponge-Stick samples that had previously collected material from real-world surfaces
- Culture and RV-PCR analysis methods did not perform as well for VFCs
- TSB enrichment of the VFC filter following spore recovery, was PCR positive at a lower loading level than RV-PCR
  - Indicates B. a. Sterne spiked onto VFC membrane are not efficiently removed from the filter
  - When TSB enrichment broth found to be positive by PCR was streaked onto SBA, B. a. Sterne was not isolated (except for field blank samples)
- RV-PCR can be used to positively identify viable *B. anthracis* in the presence of complex, dirty sample matrices from Sponge-Stick surface samples
  - Samples with as few as 15 B. a. Sterne spores (CFU) were positively identified routinely

Acknowledgements

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  - https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NHSRC&dirEntryId=346146