U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

PNNL-23094, Rev. 1

Experimental Design for a Macrofoam-Swab Study Relating the Recovery Efficiency and False Negative Rate to Low Concentrations of Two *Bacillus anthracis* Surrogates on Four Surface Materials

GF Piepel
JR Hutchison

December 2014

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Richland, Washington 99352

Executive Summary

This report describes the experimental design for a laboratory study to quantify the recovery efficiencies (REs), false negative rates (FNRs), and limits of detection (LODs) of a validated macrofoam-swab sampling method for low concentrations of *Bacillus anthracis* Sterne (BAS) and *Bacillus atrophaeus* Nakamura (BG) spores on four surface materials. The experimental design was developed at Pacific Northwest National Laboratory (PNNL), where the testing was performed.

The high-level drivers for this work were two concerns raised by the Government Accountability Office (GAO) following the 2001 contaminations of the Hart Senate Office Building and postal facilities via letters containing *Bacillus anthracis* (BA). These concerns involved (i) the lack of validated sample collection, processing, and analytical methods and (ii) insufficient use of statistical sampling to provide for quantifying the confidence that part or all of a building can be cleared when all samples have negative results (GAO 2005a, 2005b). Critical to addressing both of these areas of concern is quantifying the FNR for a single sample. The FNR for a single sample may depend on the 1) method of contaminant deposition, 2) surface concentration of the contaminant, 3) surface material being sampled, 4) sample collection method, 5) sample storage and transportation method and conditions, 6) sample processing method, and 7) sample analytical method.

A review of the literature (Piepel et al. 2012) found 20 chamber and laboratory studies that 1) collected swab, wipe, or vacuum samples from many surface materials contaminated by BA or a surrogate and 2) used culture methods to determine the surface concentration of spores as measured by colony-forming units (CFUs). With one exception, these studies quantified the performance of the sampling and analytical methods in terms of RE and not FNR. Only Krauter et al. (2012) studied low enough concentrations to support developing equations for calculating FNR as a function of surface concentration. Otherwise, Piepel et al. (2012) noted that previous chamber and laboratory studies failed to quantify the FNR for a single sample as a function of affecting variables, and this left a major gap in the information about the performance of surface sampling methods at low concentrations. Quantifying the FNR for a single sample under a variety of conditions is a key aspect of 1) validating sample and analytical methods and 2) calculating the confidence in characterization or clearance decisions based on a statistical sampling plan. These are the two main areas of concern expressed by the GAO.

The initial step to address the gap in FNR results for BA contamination was the sponge-wipe study of Krauter et al. (2012), which used BG as a surrogate for BA. The next step was to generate FNR results by performing a similar study using a validated, macrofoam-swab sampling method (Hodges et al. 2010). This report describes the experimental design that was conducted to examine the performance of the macrofoam-swab method with two BA surrogates (BAS and BG) at multiple low concentrations on four surface materials. This report also discusses the planned results and data analyses. The previous Rev. 0 of this report presented the experimental design that was initially planned, but some changes were made during the testing. Hence, this Rev. 1 of the report describes the experimental design as it was actually performed. The key response variables included 1) contamination on positive-control samples associated with test coupons, 2) contamination recovered from test coupons by swab sampling, 3) RE, 4) FNR, and 5) LOD. All of these response variables were determined using a culture method, while FNR and LOD were determined using a modified Rapid Viability-Polymerase Chain Reaction (mRV-PCR) method.

Ideally, the macrofoam-swab study would have used dry aerosol deposition of surrogates on test coupons inside a test chamber. That approach was initially investigated for the sponge-wipe study (Krauter et al. 2012), but it was not possible to reliably achieve the low concentrations of BG on test coupons necessary to obtain false negatives. Hence, the sponge-wipe study was performed as a laboratory (bench-top) study using a liquid deposition method that reliably achieved low concentrations of the contaminant on test coupons so that false negatives were achieved. For this reason, the macrofoam-swab study was conducted in a laboratory using a liquid deposition method similar to that described by Krauter et al. (2012).

The experimental design for the macrofoam-swab laboratory study was initially planned to investigate eight concentrations of each BA surrogate (BAS and BG) on 12 test coupons each of four nonporous surface materials. The four surface materials (stainless steel, glass, vinyl tile, and plastic [from a ceiling light cover panel]) were selected to span a range of surface roughness values. Test coupons were 2 inches by 2 inches. The initial plan was for two sets of eight test runs (corresponding to eight concentrations) for each of the two BA surrogates, for a total of 32 test runs. However, after the first 16 test runs, the tests at the highest two target concentrations (100 and 500 spores/coupon) yielded no false negatives. Hence, it was decided for the remaining test runs to terminate testing with the target concentration of 500 spores/coupon and substitute tests with a target concentration of 4 spores/coupon. To obtain results at this latter concentration for 12 test coupons per combination of surrogate and surface material, additional test runs were performed. Hence, the second half of testing consisted of two sets of nine test runs. During each test run, two tests using the culture and mRV-PCR analytical methods were performed. Ultimately, for each of BAS and BG, there were 68 tests in 34 test runs that were performed in four sets of eight or nine test runs. The eight or nine target concentrations of a BA surrogate for a given set of test runs were randomly assigned to the eight or nine test runs. It was expected that some of the very low concentrations might present challenges for deposition, sampling, and analysis. However, such tests were needed to obtain FNRs ranging up (or near) to one.

The experimental design for this laboratory study was a split-split-split-plot experiment (Steel and Torrie 1960, Chapter 12; Jones and Nachtsheim 2009; Kowalski et al. 2010) in which "BA surrogate" is the whole-plot (WP) factor, "concentration" is the sub-plot (SP) factor, and "analytical method" (culture and mRV-PCR) is the sub-sub-plot (SSP) factor. Six replicate coupons of each of the four surface materials were tested in each SSP, with the coupons assigned to a biosafety cabinet in a balanced manner. Over the two sets of eight or nine test runs for a BA surrogate, there were 12 test coupons for each of the four surface materials. This was a good number of replicates for fitting FNR-concentration equations for each combination of surrogate and surface material. Positive-control samples were generated by placing the same number of drops of BA surrogate solution on a growth plate (for culture) or in a collection tube (for mRV-PCR) as were placed on associated test coupons. Creating positive-control samples at the same time as test coupons provided for estimating the actual contaminant concentration deposited on each test coupon (within the variation of the deposition process). For each of the four surface materials, the actual contaminant concentrations will be estimated from the test results and used during the data-analysis phase 1) to calculate RE values for the macrofoam-swab sampling and analytical method and 2) as the concentrations for assessing relationships of RE and FNR with concentration.

Data analyses for the swab laboratory study will support:

- estimating the actual surface concentrations (e.g., CFU/cm^2) and their uncertainties (i.e., standard deviation [SD] or percent relative standard deviation [%RSD]) for each combination of BA surrogate, concentration, surface material, and analytical method,
- estimating the RE (%) and its uncertainty (SD or %RSD) for each combination of BA surrogate, concentration, and surface material for the culture analytical method,
- estimating the FNR for each combination of BA surrogate, concentration, surface material, and analytical method, and
- assessing the relationship of RE and FNR with concentration for each combination of BA surrogate, surface material, and analytical method.

Using the FNR-concentration equations that will be generated using the data from this study, it will be possible to calculate quantities such as the LOD for each combination of BA surrogate, surface material, and analytical method, as well as the predicted FNR for any concentration in the range tested. Finally, it will be possible to assess whether there are differences in results for the two BA surrogates and the two analytical methods.

In summary, the data resulting from the macrofoam-swab laboratory study discussed in this report will support addressing both of the main concerns of the GAO, namely 1) contributing information toward validating the swab method and 2) providing FNR values as a function of concentration and surface material for calculating the numbers of samples as well as confidence in characterization and clearance decisions based on statistical sampling plans when the FNR > 0.

Acronyms

%RSD	percent relative standard deviation
BA	*Bacillus anthracis*
BAS	*Bacillus anthracis* Sterne
BG	*Bacillus atrophaeus* Nakamura
BSC	biosafety cabinet
CDC	Centers for Disease Control and Prevention
CFU	colony-forming unit
DHS	U.S. Department of Homeland Security
DOE	U.S. Department of Energy
FNR	false negative rate
FPR	false positive rate
GAO	Government Accountability Office
LOD	limit of detection
mRV-PCR	modified Rapid Viability-Polymerase Chain Reaction
PBS-T	phosphate buffered saline with Tween 80
PNNL	Pacific Northwest National Laboratory
QC	quality control
RE	recovery efficiency
SD	standard deviation
SP	sub-plot
SSP	sub-sub-plot
SSSP	sub-sub-sub-plot
U.S.	United States
VSPWG	Validated Sampling Plan Working Group
WP	whole-plot

Acknowledgments

The Pacific Northwest National Laboratory (PNNL) work summarized in this report was funded by the Biological Research and Development Branch of the Chemical and Biological Division in the Science and Technology Directorate of the U.S. Department of Homeland Security (DHS). The interest and funding support of Randy Long (DHS) is gratefully acknowledged. PNNL is a multiprogram national laboratory operated for the U.S. Department of Energy by Battelle under Contract DE-AC05-76RL01830.

We also acknowledge and thank the following PNNL staff members: 1) Brent Pulsipher for his work as project manager, 2) Brett Amidan and Rachel Bartholomew, Brooke Deatherage Kaiser, and Aimee Holmes for reviewing and providing comments on drafts of Rev. 0 and Rev. 1 of this report, and 3) Maura Zimmerschied and Kathy Neiderhiser for editing, formatting, and preparing the report for publication. We also thank Laura Rose of the Centers for Disease Control and Prevention (CDC) for providing the CDC macrofoam-swab processing protocol.

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1.0 Introduction

This report discusses the experimental design for a laboratory study to quantify the recovery efficiencies (REs) and false negative rates (FNRs) of a validated macrofoam-swab sampling method (Hodges et al. 2010) for low concentrations of *Bacillus anthracis* Sterne (BAS) and *Bacillus atrophaeus* Nakamura (BG) on several surface materials. The laboratory study was needed to address gaps identified by Piepel et al. (2012) in a review of 20 previous laboratory studies that investigated the performance of swab, wipe, and vacuum sampling methods for contamination of *Bacillus anthracis* (BA) or surrogates. The experimental design in this report was developed at Pacific Northwest National Laboratory (PNNL) and the laboratory study was performed by PNNL.

The laboratory study produced data to characterize the performance of macrofoam-swab sample collection, extraction, and analysis by culture procedures reported by the Centers for Disease Control and Prevention (CDC) (Hodges et al. 2010). The macrofoam-swab sampling method is also recommended in the Validated Sampling Plan Working Group (VSPWG) sampling reference guide.[(a)] Additionally, the macrofoam-swab study evaluated a modified Rapid Viability-Polymerase Chain Reaction (mRV-PCR) method for analysis of samples using a modification of the protocol reported by Létant et al. (2011).

The swab study investigated the effects of selected test variables on several response variables. For several reasons, it was necessary to limit the number of test variables investigated in the macrofoam-swab study. The four independent variables that were varied in the study are the 1) BA surrogate (BAS, BG), 2) surface concentration of the surrogate, 3) surface material, and 4) analytical method (culture or mRV-PCR). Four nonporous surface materials (stainless steel, glass, vinyl tile, and plastic from a ceiling light cover panel) having a range of surface roughness values were chosen. Eight surface concentrations of surrogate spores (including some very low concentrations) were initially chosen for investigation. Partway through the study, the highest concentration was replaced by a very low concentration. The variables that were held constant or varied in the macrofoam-swab study are discussed further in Sections 2 and 3, respectively. The response variables for which data were collected included 1) contamination on positive-control samples associated with test coupons, 2) contamination recovered from test coupons by swab sampling, 3) RE, 4) FNR, and 5) limit of detection (LOD). All of these response variables were determined using a culture analytical method, while FNR and LOD were determined using an mRV-PCR analytical method.

The tests in the swab study were conducted according to the experimental design presented in Section 6. To limit the possibility that the effects of variables not directly of interest might be confounded with the effects of the test variables of interest, the experimental design involved randomizing some aspects of testing and data generation.

In actual contamination scenarios, before or after decontamination, concentrations of BA necessary to cause illness or symptoms may be low enough that the FNR > 0. Hence, for low concentrations, estimates of RE, FNR, and LOD for single samples taken under various sampling conditions are needed as inputs to calculate the confidence in detection or clearance decisions based on sampling and analysis

(a) *Reference Guide for Developing and Executing Bacillus anthracis Sampling Plans in Indoor Settings* (Draft, pre-decisional), Validated Sampling Plan Working Group, 2014.

results after a contamination event (Piepel et al. 2013). The macrofoam-swab study generated data to support developing FNR-concentration equations for combinations of the two surrogates, four surface materials, and two analytical methods. These FNR-concentration equations will then be available to provide inputs for calculating 1) the numbers of samples in statistically based sampling plans to provide the desired confidence for detection and clearance decisions and 2) the confidence in detection and clearance decisions given the numbers of samples in specific sampling plans.

Sections 1.1 and 1.2 present, respectively, the background and justification for the work in this report. Section 1.3 discusses the PNNL performing organizations and funding source. Section 1.4 outlines the organization of the rest of the report.

1.1 Background

In 2001, letters containing BA contaminated the Hart Senate Office Building in Washington, D.C. and postal facilities that processed the letters. This experience identified areas of concern in the procedures and methods used to characterize and clear buildings contaminated by BA. A congressional inquiry and the Government Accountability Office (GAO) identified two main concerns (GAO 2005a, 2005b). One main concern was the reliance on sampling specific areas where it was thought BA would be found. This type of sampling approach is referred to as *targeted sampling* or *judgmental sampling*. The GAO reports identified the need to use *statistical (probabilistic) sampling* so that when all results are negative, a building (or area within a building) can be cleared with a known level of statistical confidence. The second main concern was that the sample collection, processing, and analytical methods used were not validated. The lack of validated methods raised questions about the reliability of the negative sampling results.

The VSPWG was formed in July 2006 in response to the congressional inquiry and GAO reports. The VSPWG is headed by the U.S. Department of Homeland Security (DHS) Science and Technology Directorate and includes experts from the CDC, the U.S. Environmental Protection Agency, the U.S. Department of Defense, and the Federal Bureau of Investigation. Although not formally members of the VSPWG, staff from the National Institute of Standards and Technology and U.S. national laboratories (PNNL and Sandia National Laboratories) have played key roles. The VSPWG is developing a sampling reference guide for indoor contamination[(a)] and is working toward validating methods for steps in the sampling process. These steps include 1) sampling approach (e.g., appropriate uses of judgmental and probabilistic sampling), 2) sample collection methods, 3) handling of samples (e.g., handling, storage, and transportation), 4) sample processing and extraction methods (i.e., extraction of the contaminant from samples), and 5) sample analysis (i.e., analytical methods).

1.2 Justification

The results of the macrofoam-swab study conducted using the experimental design in this report will contribute to addressing both of the GAO's main concerns. Specifically, for macrofoam-swab samples collected, extracted, and analyzed using the selected methods, the study will yield equations relating the

(a) *Reference Guide for Developing and Executing Bacillus anthracis Sampling Plans in Indoor Settings* (Draft, pre-decisional), Validated Sampling Plan Working Group, 2014.

FNR to the surface concentration of a BA surrogate for combinations of BA surrogate, surface material, and analytical method. The four surface materials tested in the macrofoam-swab study are expected to span a range of surface material characteristics for nonporous surfaces. This may provide the basis for assessing the FNR as a function of the BA surrogate concentration for additional nonporous surface materials as a function of surface material characteristics (e.g., surface roughness).

The FNR equations will be developed from data generated by the study conducted according to the experimental design documented in this report. These equations (and the underlying data) will make a significant contribution to the validation of macrofoam-swab sample collection, extraction, and analytical methods by quantifying the RE and FNR performance for low concentrations of two BA surrogates over a range of surface materials sampled by a macrofoam swab and analyzed by one of two analytical methods. The CDC-validated method for macrofoam-swab sampling and extraction will be used with the CDC-validated culture analytical method and with an mRV-PCR analytical method. Hence, this work will address one of the two main concerns of the GAO, namely method validation.

The FNR equations will also serve as inputs to formulas for calculating the 1) numbers of samples required to make characterization and clearance decisions with specified levels of confidence and 2) confidence in characterization and clearance decisions for specific sampling plans implemented in BA contamination events. The work to develop these formulas is part of another task in the PNNL project supporting the VSPWG, which is being documented in separate reports (the first of which is Piepel et al. 2013). The experimental design for the swab study discussed in this report will generate key data on the FNR for a single swab sample. Clearly, the FNR for a single swab sample will depend on the (i) concentration of the contaminant, (ii) surface material being sampled, and (iii) sampling, extraction, and analytical processes. The FNR for a single sample is a key input to calculations for the confidence in characterization and clearance decisions based on statistical sampling. Hence, this work contributes to addressing the other main GAO concern—having confidence in decisions based on statistical sampling.

The need for the macrofoam-swab study discussed in this report was partly motivated by a PNNL review of the literature on previous laboratory studies (Piepel et al. 2012). The literature review focused mainly on studies that collected swab, wipe, or vacuum samples from surfaces contaminated by BA or a surrogate and used culture methods to determine the surface concentration of the contaminant. A total of 20 studies were identified, with 13 studies having swab data, 12 studies having wipe data, and 5 studies having vacuum data. Piepel et al. (2012) noted that quantifying the FNR in a single sample as a function of contaminant concentration, surface material, and other impacting factors was a major gap in the previous laboratory studies. Krauter et al. (2012) addressed this gap for the sponge-wipe method. The macrofoam-swab study (whose experimental design is presented in this report) will address this gap for the CDC-validated swab collection, extraction, and analytical methods in addition to mRV-PCR analysis (which is not part of CDC methods).

1.3 Staff and Funding

The work discussed in this report was performed by staff in the Applied Statistics and Computational Modeling group and the Chemical and Biological Signature Science group at PNNL. The work was funded by the Chemical and Biological Research and Development Branch of the Chemical and Biological Division of DHS. The work is under the prime contract DE-AC05-76RL01830 between the U.S. Department of Energy (DOE) and the operator of PNNL for research, testing, evaluation, and/or

development activities and pursuant to Section 309(a)(1)(c) of the Homeland Security Act of 2002 (Public Law 107-296), which authorizes DHS to task the DOE national laboratories on a "work for others" basis.

1.4 Report Organization

The remainder of this report describes the experimental design for the macrofoam-swab study and the basis for its development. The report is organized as follows. The factors that were held constant in the experimental design are discussed in Section 2. The factors that were varied in the experimental design are discussed in Section 3. The quality control (QC) samples (i.e., positive and negative controls) included in the macrofoam-swab study are discussed in Section 4. Preliminary tests that were used to determine the surface concentration levels of the surrogate contaminant are discussed in Section 5. The experimental design for the swab study is presented and discussed in Section 6. All of the gaps in previous laboratory studies identified by Piepel et al. (2012) could not be filled in this swab study, so the advantages and disadvantages of the study are discussed in Section 7. The data analyses that will be supported by the chosen experimental design are described in Section 8. Recommendations for future studies are presented in Section 9. The references cited in the report are listed in Section 10.

2.0 Factors Held Constant in the Macrofoam-Swab Study

Table 2.1 lists the factors held constant during the macrofoam-swab study.

Table 2.1. Factors Held Constant in the Macrofoam-Swab Study

Factor	Test Level
Sample collection medium	Macrofoam swab[(a)]
Sample/Coupon size	2 inches by 2 inches (25.806 cm^2)
Wetting agent	PBS-T[(b)]
Interfering material	None (clean surfaces)
Deposition	Wet deposition
Storage/transportation	None
Laboratories	One (PNNL)[(c)]
Preparation/extraction	Per method[(a)]

(a) The swab sample collection and analytical methods are based on methods developed by the CDC and will be documented in a subsequent PNNL report on the study results.
(b) PBS-T = phosphate buffered saline with Tween-80.
(c) Lab-to-lab variation is typically a major contributor to reproducibility uncertainty, which ideally would be estimated via tests at several laboratories. However, the available funding was not sufficient to use different laboratories as part of the study.

The macrofoam swab used for the study is described by Hodges et al. (2010). Test coupons that are 2 inches by 2 inches were used. A liquid deposition method similar to that used by Krauter et al. (2012) was used to deposit spores on test coupons and positive-control samples. Preliminary testing showed that this liquid deposition method reliably achieved lower concentrations of the contaminants on test coupons, so that adequate numbers false negatives would be obtained.

Preparation/extraction methods, storage/transportation factors, and analytical laboratories were held constant in this swab study. The summary of laboratory studies (Piepel et al. 2012) identified 1) preparation/extraction factors to be very important to sampling efficiency and 2) little information in the literature about storage/transportation and laboratory effects. However, varying these factors in addition to the factors selected for varying in this test (discussed in Section 3) would have comprised a very large study and was beyond the scope of this effort. It is envisioned that these factors could be investigated in future laboratory studies if deemed necessary.

3.0 Factors Varied in the Macrofoam-Swab Study

Table 3.1 lists the factors varied in the macrofoam-swab study. Each factor is discussed briefly in the following paragraphs.

Two BA surrogates (BAS and BG) were tested. Based on the literature review of Piepel et al. (2012), it appears that BAS and BG have not both been previously tested in the same study. Testing both surrogates in this study provides for comparing test results from the widely used BG surrogate with those of the BAS surrogate (which may be more representative of results with actual BA spores).

Four nonporous surface materials were tested: stainless steel, glass, vinyl tile, and plastic (from ceiling light cover panels). These materials were selected to span a range of surface roughness of nonporous materials (Shellenberger and Logan 2002, Hallas and Shaw 2006, Krauter et al. 2012). It was considered more important to span a range of surface roughness values than to select surface materials that would normally be sampled by a macrofoam swab (many such surfaces are very smooth). Spanning the range of surface roughness values will allow applying the results of the macrofoam-swab study to various surface materials that might be sampled by a swab.

Table 3.1. Factors Varied in the Macrofoam-Swab Study

Factor	Test Levels
Bacillus anthracis surrogate	*Bacillus atrophaeus* Nakamura (ATCC 9372), *Bacillus anthracis* Sterne
Surface material	Stainless steel, glass, vinyl tile, and plastic (from a ceiling light cover panel)
Surrogate surface concentration	Eight (subsequently nine) different concentrations (determined from preliminary scoping tests—see Section 5)[(a)]
Number of test coupons per surface material	12 test coupons for each of the four surface materials
Analytical method	Culture, mRV-PCR
Biosafety cabinet[(b)]	Two Nuaire Class II, Type A2 6-foot biosafety cabinets (BSCs) were used to enable faster progress in testing.
Personnel for sample collection, processing, and analytical[(b)]	Two personnel per test run to perform the sample collection, extraction, and analytical steps of the work

(a) The original eight concentrations were determined by preliminary, range-finding scoping tests that were conducted prior to the actual test matrix (see Section 5). Halfway through testing, the largest concentration was replaced by a next-to-lowest concentration, for a total of nine concentrations.

(b) The effects of these factors are not directly of interest, but they were varied/controlled in the experimental design so that their effects were not confounded with the effects of factors that are directly of interest.

Eight different surface concentrations were chosen to span the range of FNR values (0 to 1) for the four surface materials. The process for selecting the eight concentrations to use in tests with each BA surrogate and each analytical method is discussed in Section 5.1. The number of concentrations was chosen to protect against the possibility that the 1) lowest two concentrations yield FNR ≈ 1, and 2) highest two concentrations yield FNR ≈ 0. In such a case, there would be four concentrations to span the interior of the 0 to 1 range for FNR. However, it would be better to have six such concentrations with only one concentration each yielding FNR ≈ 0 and FNR ≈ 1. In the first half of testing, the highest two concentrations yielded no false negatives for either surrogate. Hence, it was decided to replace the highest concentration with a second-lowest concentration in the remaining tests. Thus, a total of nine concentrations were tested. The details are discussed subsequently.

There were 12 test coupons for each surface material for each combination of BA surrogate, concentration, and analytical method. Having 12 coupons for each surface material was reasonable for the test budget and provided a good basis for estimating FNR values and fitting FNR-concentration equations. Positive and negative control samples included in the experimental design are discussed in Sections 4 and 6.

Two analytical methods were used to produce test results: culture and mRV-PCR. In the previous sponge-wipe study (Krauter et al. 2012), only the culture method was used. It was decided to also include the mRV-PCR method in this macrofoam-swab study because of the small additional cost to do so.

Two additional factors that are not specifically of interest, but might affect test results, were varied in the study, as follows.

- To permit completion of testing in a reasonable time, two BSCs were used. The combinations of settings of the test factors of interest were assigned to BSCs in a balanced way so that any possible difference between the two BSCs would not be confounded with the effects of the test factors of interest. Also, preliminary tests discussed in Section 5.2 were performed to assess whether there was any difference in test results using the two BSCs.
- Two persons performed the sample collection, extraction, and analytical (i.e., culture or mRV-PCR) steps of the sampling and analytical work. Which person performed each task in the process was controlled to avoid confounding the effects of personnel with those of the test factors of interest. Also, varying the personnel performing the tests includes a source of reproducibility uncertainty in the test results (which often is not done in such studies, see Piepel et al. [2012]).

Section 6 discusses in more detail how all of the preceding factors were varied as part of the experimental design.

4.0 Quality Control Samples in the Macrofoam-Swab Study

Each test run in the macrofoam-swab study included a complement of QC samples, which were thoroughly documented in the PNNL test procedures. The QC samples comprise positive and negative control samples.

All test coupons within a test run (i.e., the pair of tests in the two BSCs with one analyzed by culture and the other by mRV-PCR) had the same target concentration of BA surrogate, which was implemented with a specified number of drops of the BA surrogate solution. Hence, positive controls received the same number of drops of the solution as test coupons. The number of drops for positive controls was placed

- on a growth media plate for culture analysis (each test)
- in a collection tube for DNA extraction and mRV-PCR analysis (only tests for mRV-PCR analysis).

The positive controls will provide for 1) quantifying the REs associated with the swab sampling and analytical method and 2) relating FNRs to BA surrogate concentrations for each combination of the two BA surrogates, eight concentrations, four surface materials, and two analytical methods (culture, mRV-PCR). Note that the mRV-PCR results will involve relative quantification based on the starting cell concentration and a DNA standard curve.

The negative controls consisted of 1) blank coupons that were processed the same as spore-seeded coupons and 2) blank swabs (process negatives) that were taken out of their packages, placed in the appropriate specimen containers, and processed the same as swab samples of contaminated coupons. A positive result on any negative control sample associated with a test would invalidate the associated tests, which would require them to be performed again. However, positive results on negative control samples were documented because they provide for estimating the false positive rate (FPR) of the macrofoam-swab method.

Additional details regarding the positive and negative controls are discussed in Section 6 and were provided in the PNNL procedures for the experimental work.

5.0 Preliminary Tests

Section 5.1 discusses the preliminary tests that were performed to determine the concentrations of the two BA surrogates (BAS, BG) that were used in actual testing. Section 5.2 discusses the preliminary tests that were conducted to assess the effects of secondary factors that were varied in the experimental design. Section 5.3 discusses the preliminary tests performed to optimize mRV-PCR detection.

5.1 Preliminary Tests to Determine Contaminant Concentrations

Preliminary "range finding" tests were performed before the actual tests in the macrofoam-swab study to determine the eight surface concentration levels that would be used for testing. The set of eight concentrations of the BA surrogates (BAS, BG) was selected with the goal that (i) the lowest concentration is the largest for which FNR ≈ 1, (ii) the highest concentration is the smallest for which FNR ≈ 0, and (iii) intermediate concentrations have intermediate FNR values spanning the 0 to 1 range. The data from the test runs will be used to fit an FNR-concentration curve for each combination of BA surrogate, surface material, and analytical method.

Figure 5.1 shows an example of the goal for choosing the concentration levels, taking into account the four surface materials (stainless steel, glass, vinyl tile, and plastic from a ceiling light cover panel). The four colored curves in Figure 5.1 represent a hypothetical relationship between FNR and concentration for each of four surface materials. If the four curves in Figure 5.1 would be substantially different for different combinations of BA surrogate and analytical method, then the set of eight concentrations selected would have to be different. However, it turned out that the same set of concentrations could be used for all tests.

The set of eight concentrations were chosen so no more than two of the concentrations (for any of the four surface materials) would yield FNR values equal or very close to 0 or to 1. For example, suppose for a given surface material that the two smallest concentrations (#1 and #2 in Figure 5.1) yield FNR = 1, while the two largest concentrations (#7 and #8 in Figure 5.1) yield FNR = 0. That would leave four concentrations to yield FNR values with intermediate values of $0 < FNR < 1$. Four concentrations with intermediate FNR values would be the minimally acceptable basis (during the data analysis phase of work described in Section 8) to fit sigmoidal equations to the FNR-concentration data. Ideally, for each surface material, six of the eight concentrations would yield intermediate values of FNR, with only the smallest concentration yielding FNR = 1, and only the largest concentration yielding FNR = 0.

In summary, the eight concentrations initially tested in the swab study were chosen based on preliminary/scoping tests conducted before the actual tests. Because the two highest concentrations yielded no false negatives for any combination of surrogate, surface material, and analytical method, the highest concentration was replaced with a new, second-lowest concentration halfway through testing. The details are discussed in Section 6.3.

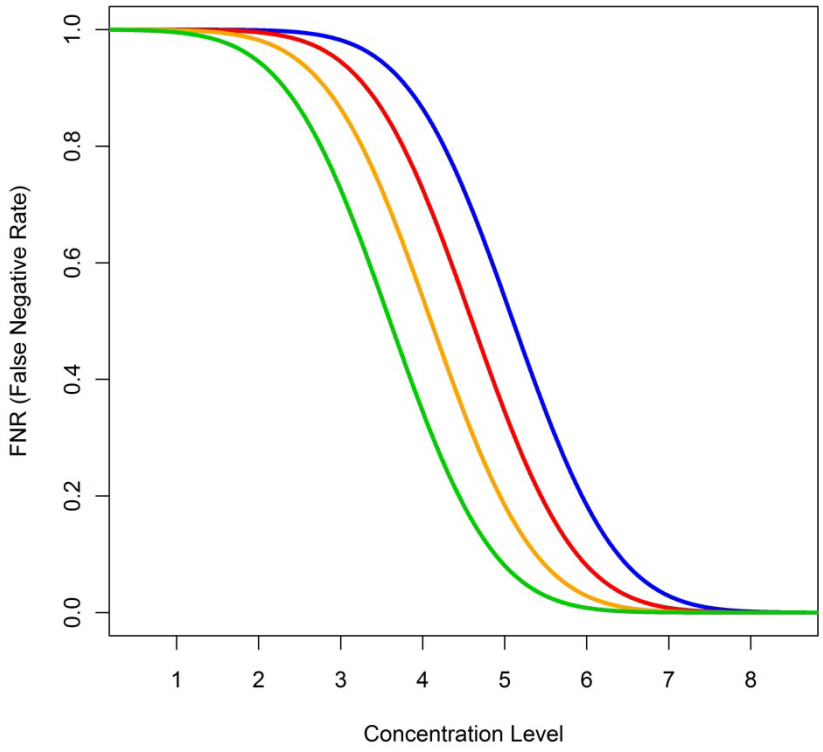

Figure 5.1. An Example of Eight Concentration Levels Selected to Span the Range of FNR Values for Four Surface Materials. The four curves represent hypothetical relationships between FNR and concentration for the four surface materials.

5.2 Preliminary Tests to Assess Other Experimental Variables

Preliminary tests were also conducted prior to the actual tests to assess the following experimental factors that might impact the experimental design.

- Two Nuaire Class II, Type A2 BSCs (which are adjacent in the same laboratory) were used for testing. Preliminary tests were conducted to assess whether there is any systematic difference in results from the two BSCs.
- Tests were conducted with test coupons and positive-control samples at various test locations within the planned work area of a BSC. Preliminary tests were used to assess whether test results depend on the location of a sample within a BSC.
- The experimental design discussed subsequently in Section 6 assumes that surrogate spores will not migrate from test coupons or positive-control samples to other locations within a BSC. This is assumed because of the liquid deposition and drying procedure that will be used. Preliminary tests were conducted to assess this assumption.

The results of preliminary testing showed that there were no statistically significant effects due to BSC and location within a BSC. Further, there was no evidence of migration of spores within a BSC. The experimental design of the swab study discussed in Section 6 was hence constructed assuming 1) no systematic differences in results between the two BSCs, 2) no location effects for the planned layout of

test coupons and positive-control samples (discussed in Section 6), and 3) no migration of surrogate spores within a BSC.

5.3 Preliminary Tests to Optimize mRV-PCR Detection

Additional preliminary tests were conducted to optimize mRV-PCR detection and relative quantification. The results of the preliminary testing did not affect the experimental design, but were pertinent to 1) sample extraction and 2) sample detection.

- Preliminary tests were conducted to determine the most efficient sample-preparation and DNA-extraction protocols. Several publications compare various commercial DNA extraction protocols for BA spores, but these protocols are designed for large numbers (e.g., 10E+7) of spores. Preliminary tests were used to determine whether PCR could be conducted directly on spores or an enrichment step was needed to recover enough DNA for detection.
- Preliminary tests using published mRV-PCR primers and probes were conducted to down-select a primer-and- probe set that is 1) robust and 2) has a low LOD.

The preliminary tests to optimize mRV-PCR conditions elucidated the optimum methods for sample preparation and mRV-PCR detection.

6.0 Experimental Design for the Macrofoam-Swab Study

This section discusses the experimental design of the swab study. Section 6.1 discusses the restrictions on randomization that applied in conducting the swab study. It also introduces the statistical terminology for experiments with restrictions on randomization. Section 6.2 discusses how the tests were conducted in two BSCs as well as the layout of test coupons, positive controls, and negative controls in the BSCs. Section 6.3 provides the details of the test matrix for the experimental design.

6.1 Restrictions on Randomization in Conducting the Macrofoam-Swab Study Tests

The simplest structure of test data is obtained when all tests are performed in a random order. However, in practice it can be too time consuming or impractical to completely randomize the order of all tests. In practice, one or more restrictions on randomization may occur. For the macrofoam-swab study, there were three restrictions on randomization:

- It would have been too complicated and would have risked cross-contamination to randomly intermix tests with the two BA surrogates. Tests with a given BA surrogate were performed in four sets (BAS, BG, BAS, BG) containing 16, 16, 18, and 18 tests, respectively.
- After a given concentration of a BA surrogate was prepared, all tests with that concentration were performed as a set.
- Finally, it simplified matters to conduct together in subsets the tests with a given combination of BA surrogate and concentration to be analyzed by culture (in one BSC) and mRV-PCR (in the other BSC).

For each combination of BA surrogate, concentration, and analytical method, a total of 12 replicate test coupons of each of the four surface materials was tested. The experimental design for the swab study involves three restrictions on randomization, so it is referred to as a *split-split-split-plot experiment* (Steel and Torrie 1960, Chapter 12; Jones and Nachtsheim 2009; Kowalski et al. 2010). The split-split-split-plot and randomization aspects of the experimental design are further described in the following paragraphs.

In the terminology of experiments with restrictions on randomization, "BA surrogate" is the whole-plot (WP) factor, "concentration" is the sub-plot (SP) factor, "analytical method" (culture and mRV-PCR) is the sub-sub-plot (SSP) factor, and "surface material" is the sub-sub-sub-plot (SSSP) factor.[(a)] The WP, SP, and SSP factors each involve a restriction on randomization as described above, which means there are four components of experimental and analytical variance instead of one (if there were no restrictions on randomization). Also, the restrictions on randomization induce covariances among subsets of the resulting data. This structure of the data must be accounted for in designing the experiment and in analyzing the data (Jones and Nachtsheim 2009, Kowalski et al. 2010).

(a) The split-plot, whole-plot, sub-plot, etc. terminology comes from the original application of split-plot experimental designs in agricultural field trials where plots of land were split, possibly more than once. The terminology has been retained in the statistics literature, even though split-plot experiments are widely used in many application areas and often periods of time are split rather than plots of land.

For a given set of tests with a BA surrogate, the eight concentrations were assigned randomly to the eight test runs (nine test runs for the second half of testing) for each of the two analytical methods (culture, mRV-PCR) to protect against confounding any possible time-trend effects with concentration effects over the eight (or nine) test runs. Over all tests for a given BA surrogate, the four nonporous surface materials were investigated with 12 test coupons for each material. Having 12 test coupons per material was reasonable from a test budget perspective, and provides a good basis for fitting FNR-concentration equations to the data.

6.2 Locations for Test Coupons, Positive Controls, and Negative Controls in the Biosafety Cabinets

The contaminant deposition and sample collection aspects of the testing were performed in two Nuaire Class II, Type A2 BSCs, each of which has a working area 70 inches wide and 18 inches deep. Although use of a BSC is only necessary for BAS and not BG, a BSC was used for the consistency of testing both BA surrogates.

The numbers and placements of test coupons, positive-control samples, and negative control samples within the BSCs are an important part of the experimental design for the macrofoam-swab study. The results for test coupons and positive controls will provide for 1) quantifying the REs associated with the swab sampling and analytical methods and 2) relating FNRs to BA surrogate concentrations for each combination of BA surrogate, surface material, and analytical method. Section 4 describes how positive-control samples were generated for both the culture and mRV-PCR analytical methods.

Ideally, a positive control would be colocated next to each test coupon within each BSC, so that there would be a one-to-one correspondence between test coupons and positive controls. Doing that would provide for assessing during actual tests whether there are any 1) differences in results from the two BSCs that will be used for testing and 2) location effects within the BSCs. If there were such differences or effects, having a positive-control sample colocated with each test coupon provides for adjusting for such differences or effects in statistical analyses of the data.

However, the space inside the BSCs is limited. It was initially judged ideal to have two rows (each with 12 locations) within a BSC with one row for test coupons and the other row for positive-control samples. Further, it was envision having the two rows located in the middle (front to back) of a BSC to avoid having samples located near the front or back of a BSC. Such a layout would only provide for 12 test coupons and 12 positive-control samples per BSC run if one positive control was paired with each test coupon. This layout would have required four BSC runs to complete the 48 test coupons and 48 positive-control samples for each combination of BA surrogate, concentration, and analytical method. However, this layout was judged to require too many BSC runs and too much time/resources.

To reduce the number of BSC runs, it was decided to use an arrangement of three rows, each with 12 locations, for test coupons and positive-control samples within a BSC. The second (middle) of the three rows contained positive-control samples, while the other two rows contained test coupons (see Figure 6.1). Hence, only two BSC runs (each containing 24 test coupons and 12 positive controls) were required to complete the 48 test coupons for each combination of BA surrogate, concentration, and analytical method. The reduction of BSC runs from four to two per combination was at the cost of having half the number of positive-control samples. Hence, rather than having positive-control samples paired

one-for-one with test coupons, each positive-control sample in the second (middle) row could be associated with the two test coupons on either side of it in the first and third rows. This was deemed acceptable, because the preliminary tests discussed in the second bullet of Section 5.2 showed that there are no location effects among the three rows of 12 samples in a BSC. Hence, it was not be necessary to have a one-to-one relationship between positive-control samples and test coupons.

Four coupon negative controls were located within a BSC on the far right. The needed consumables (tips) and equipment (trash, pipettor, tips, and stir plate) were located on the far left. Coupons were placed in sterile petri dishes to allow for ease of setup.

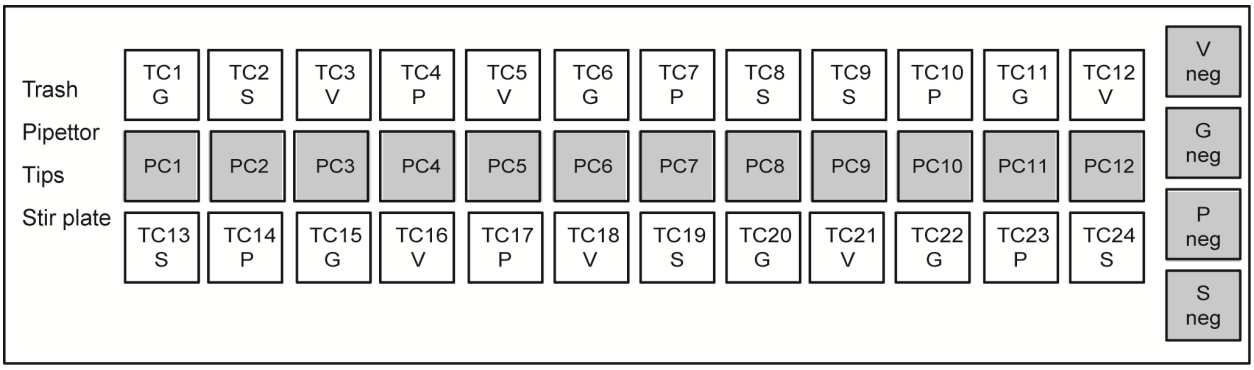

Figure 6.1. Testing Configuration in a Biosafety Cabinet. Shown are the locations of the 24 Test Coupons (TC1 – TC24; white fill), the 12 Positive Controls (PC1 – PC12; gray fill), and the four negative coupon controls (neg; gray fill) for each test. The surface materials are denoted by G = glass, S = stainless steel, V = vinyl tile, and P = plastic light cover panel. Additionally, the needed consumables and equipment (trash, pipettor, tips, and stir plate) were located to the left of the test coupons. The working surfaces of the BSCs and coupon locations are not drawn to scale and are a pictorial representation only.

6.3 Test Matrix for the Macrofoam-Swab Study

Before the testing began, the experimental design called for testing each BA surrogate twice, with eight test runs (concentrations) each time, for a total of $4 \times 8 = 32$ test runs. With two tests per test run (one each for the two analytical methods), a total of 64 tests were initially planned. Each of these tests corresponds to a combination of BA surrogate, concentration, and analytical method. These tests were initially planned to be conducted at the eight target concentrations of 2, 5, 10, 15, 20, 25, 100, and 500 spores/coupon, as determined from preliminary tests that were conducted as discussed in Section 5.1.

After completing the first half of the tests (the first 16 test runs), there were (i) no false negatives at target concentrations of 500 spores/coupon, and (ii) no or very few false negatives at the various test combinations at the next lower concentration of 100 spores/coupon. This result reduced the value of tests at 500 spores/coupon. It was decided that in the second half of testing, the tests at 500 spores/coupon would be replaced by tests at 4 spores/coupon. The choice of 4 spores was a compromise in that the data from the first half of testing showed that some test combinations would benefit most (in terms of fitting FNR-concentration functions) from an additional concentration between 2 and 5 spores/coupon, while other test combinations would benefit most from an additional concentration between 5 and 10 spores/coupon. Because 1) more test combinations would benefit from an additional concentration between 2 and 5 spores/coupon and 2) the FNR results for replicate coupons of surface materials were the

most variable (i.e., uncertain) for 2 and 5 spores/coupon, a value between 2 and 5 was chosen. The choice of 4 spores/coupon was made because it would be of more help than a smaller value (3 spores/coupon) for those test combinations that would have benefited most by an additional concentration between 5 and 10 spores/coupon.

Finally, to obtain data for 12 replicate coupons of each surface material at a concentration of 4 spores/coupon, it was necessary to include two additional test runs in the second half of testing (one each for the BAS and BG surrogates). That is because the original experimental design called for the first half of testing to obtain data on 6 replicate coupons per surface material, and the second half of testing to obtain data on an additional 6 replicate coupons per surface material. To obtain data for 12 test coupons with a target concentration of 4 spores/coupon, it was necessary to test that concentration in two test runs (for a total of 9 rather than 8 test runs) for the final sets of tests with BAS and BG. Hence, the final experimental design involved a total of 68 tests in 34 test runs.

Table 6.1 lists the general aspects of the split-split-split-plot experimental design for the macrofoam-swab study. The first column of Table 6.1 lists the *test run* numbers (1–34), where a test run involves two tests (one in each of the two BSCs) with one analytical method applied to the tests in one BSC, and the other analytical method applied to the tests in the other BSC. A test run corresponds to a given combination of BA surrogate and concentration.

The second column of Table 6.1 lists the test numbers 1–68, which also represent the run order of the tests. The run order includes randomization within the limits of the restrictions on randomization, as discussed subsequently.

The third column of Table 6.1 lists the BA surrogate used in each test run, where there were four sets of test runs of the BA surrogates in the order BAS, BG, BAS, BG. The first two sets contained 16 test runs each, while the last two sets contained 17 test runs each (because of the need to perform an additional test run [i.e., two total] with 4 spores/coupon). Testing the surrogates twice each in two sets of test runs instead of once each in one set of test runs was necessary to be able to statistically assess the effect of BA surrogate on results, because of the restriction on randomization associated with the BA surrogates (as discussed in Section 6.1). Because each BA surrogate was tested in two different sets of tests, the working spore stock for each nominal concentration was made twice for each surrogate. The actual concentrations of working spore stock may not be exactly the same as the target values in the two sets of tests for a BA surrogate, but that is acceptable and is what provides for statistically comparing the results for the BA surrogates. However, only testing each surrogate twice will provide a very limited basis for statistically comparing test results for the two surrogates. This is a necessary limitation, because the budget for testing did not allow for testing the surrogates more than twice each.

The fourth column of Table 6.1 lists the target concentration for each test. The target concentrations were pseudo-randomly assigned to the test runs, as shown in Table 6.1. The concentrations were assigned to the test runs in a structured (yet pseudo-random) way to provide for assessing test results for initial concentrations, and modifying the concentrations scheduled to be tested subsequently if needed, so as to be able to obtain the desired FNR data (as discussed in Section 5.1). The pseudo-random assignment protected against any kind of trends in results that may have occurred over time, so that such effects were not confounded with the effects of concentration on the response variables.

Table 6.1. Test Matrix for the Split-Split-Split-Plot Experimental Design of the Macrofoam-Swab Study[a]

Test Run	Test[b]	BA Surrogate[c]	Concentration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons				# Positive Controls[g]	# Negative Controls		Testing Task[j]		
						Stainless Steel	Glass	Vinyl Tile	Plastic		Coupons[h]	Swabs[i]	A	B	C
1	1	BAS	20	C	BSC1	6	6	6	6	12	4	4	2	1	2
	2	BAS	20	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
2	3	BAS	25	C	BSC2	6	6	6	6	12	4	4	1	2	1
	4	BAS	25	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
3	5	BAS	2	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	6	BAS	2	C	BSC1	6	6	6	6	12	4	4	1	2	1
4	7	BAS	10	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	8	BAS	10	C	BSC2	6	6	6	6	12	4	4	2	1	2
5	9	BAS	5	C	BSC1	6	6	6	6	12	4	4	2	1	2
	10	BAS	5	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1
6	11	BAS	100	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	12	BAS	100	C	BSC2	6	6	6	6	12	4	4	2	1	2
7	13	BAS	15	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	14	BAS	15	C	BSC2	6	6	6	6	12	4	4	1	2	1
8	15	BAS	500	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	16	BAS	500	C	BSC2	6	6	6	6	12	4	4	1	2	1
9	17	BG	20	C	BSC2	6	6	6	6	12	4	4	1	2	1
	18	BG	20	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
10	19	BG	25	C	BSC1	6	6	6	6	12	4	4	2	1	2
	20	BG	25	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1
11	21	BG	2	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	22	BG	2	C	BSC2	6	6	6	6	12	4	4	1	2	1
12	23	BG	10	C	BSC2	6	6	6	6	12	4	4	2	1	2
	24	BG	10	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
13	25	BG	5	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	26	BG	5	C	BSC1	6	6	6	6	12	4	4	2	1	2
14	27	BG	100	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	28	BG	100	C	BSC2	6	6	6	6	12	4	4	2	1	2
15	29	BG	15	C	BSC1	6	6	6	6	12	4	4	1	2	1
	30	BG	15	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2

Table 6.1. Test Matrix for the Split-Split-Split-Plot Experimental Design of the Macrofoam-Swab Study (contd)

Test Run	Test[b]	BA Surrogate[c]	Concentration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons				# Positive Controls[g]	# Negative Controls		Testing Task[j]		
						Stainless Steel	Glass	Vinyl Tile	Plastic		Coupons[h]	Swabs[i]	A	B	C
16	31	BG	500	C	BSC2	6	6	6	6	12	4	4	2	1	2
	32	BG	500	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
17	33	BAS	20	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	34	BAS	20	C	BSC1	6	6	6	6	12	4	4	2	1	2
18	35	BAS	4	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
	36	BAS	4	C	BSC1	6	6	6	6	12	4	4	1	2	1
19	37	BAS	25	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	38	BAS	25	C	BSC2	6	6	6	6	12	4	4	1	2	1
20	39	BAS	2	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	40	BAS	2	C	BSC2	6	6	6	6	12	4	4	2	1	2
21	41	BAS	10	C	BSC1	6	6	6	6	12	4	4	1	2	1
	42	BAS	10	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
22	43	BAS	5	C	BSC1	6	6	6	6	12	4	4	1	2	1
	44	BAS	5	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
23	45	BAS	100	C	BSC1	6	6	6	6	12	4	4	2	1	2
	46	BAS	100	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
24	47	BAS	15	C	BSC2	6	6	6	6	12	4	4	1	2	1
	48	BAS	15	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
25	49	BAS	4	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
	50	BAS	4	C	BSC1	6	6	6	6	12	4	4	1	2	1
26	51	BG	20	C	BSC2	6	6	6	6	12	4	4	2	1	2
	52	BG	20	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
27	53	BG	4	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	54	BG	4	C	BSC1	6	6	6	6	12	4	4	1	2	1
28	55	BG	25	C	BSC1	6	6	6	6	12	4	4	1	2	1
	56	BG	25	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
29	57	BG	2	mRV-PCR	BSC2	6	6	6	6	12	4	4	2	1	2
	58	BG	2	C	BSC1	6	6	6	6	12	4	4	1	2	1
30	59	BG	10	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	60	BG	10	C	BSC2	6	6	6	6	12	4	4	1	2	1
31	61	BG	5	C	BSC1	6	6	6	6	12	4	4	2	1	2
	62	BG	5	mRV-PCR	BSC2	6	6	6	6	12	4	4	1	2	1

Table 6.1. Test Matrix for the Split-Split-Split-Plot Experimental Design of the Macrofoam-Swab Study (contd)

Test Run	Test[b]	BA Surrogate[c]	Concentration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons: Stainless Steel	# Test Coupons: Glass	# Test Coupons: Vinyl Tile	# Test Coupons: Plastic	# Positive Controls[g]	# Negative Controls: Coupons[h]	# Negative Controls: Swabs[i]	Testing Task[j]: A	Testing Task[j]: B	Testing Task[j]: C
32	63	BG	100	C	BSC2	6	6	6	6	12	4	4	1	2	1
	64	BG	100	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
33	65	BG	15	mRV-PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	66	BG	15	C	BSC2	6	6	6	6	12	4	4	1	2	1
34	67	BG	4	mRV-PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	68	BG	4	C	BSC2	6	6	6	6	12	4	4	1	2	1

(a) Specifics of the test matrix for each test run not shown in this table are given in Figure 6.1.
(b) The Test number also represents the Run Order number of the tests.
(c) BAS = *Bacillus anthracis* Sterne, BG = *Bacillus atrophaeus* Nakamura.
(d) The concentrations are the target numbers of spores per coupon or positive-control sample, where the coupons are 2 in. × 2 in. (25.806 cm^2). The concentrations were assigned to test runs in a pseudo-random manner.
(e) C = culture, mRV-PCR = modified Rapid Viability-Polymerase Chain Reaction. The two analytical methods were randomly assigned to BSC1 and BSC2 for each pair of tests in a test run at the same concentration, such that each analytical method is used the same number of times within the sets of Tests 1–16, 17–32, 33–50, and 51–68.
(f) Two BSCs were located adjacent to each other on one wall of a laboratory. The left one was designated BSC1 and the right one was designated BSC2. The two BSCs ran tests simultaneously, with the one started first in each pair of tests determined randomly.
(g) There was one positive-control sample associated with the two test coupons on either side of it as shown in Figure 6.1.
(h) For each test in a BSC, there was one blank (uncontaminated) coupon of each of the four surface materials, for a total of four negative coupon controls.
(i) For each test in a BSC, there was four blank swabs, removed from their packages and immediately placed in the same kind of sample containers used for swabs that sampled contaminated coupons.
(j) Two persons performed the testing tasks. These tasks were: (A) sample deposition, (B) sample collection and extraction, and (C) sample analysis. The numbers 1 and 2 represent the specific person assigned to perform each task for each test. For logistics reasons, the person who performed Task A also performed Task C. Persons 1 and 2 were randomly assigned to Tasks A and B for each test, such that each person performed Tasks A and B eight times within the sets of Tests 1–16, 17–32, 33–48, and 51–66. The extra tests (49-50 and 67-68) at a target concentration of 4 spores/coupon had the persons assigned separately.

The fifth column of Table 6.1 identifies which of the two analytical methods (culture, mRV-PCR) was used for each test. All tests in one of the BSCs were analyzed with one method, while all of the tests in the other BSC were analyzed with the other method. The analytical method used in each BSC for a given test run was randomly assigned.

The sixth column of Table 6.1 lists which of the two BSCs was used for each test. A pair of tests was conducted at essentially the same time in the two BSCs, although one had to be started before the other. The BSC that was started first for each pair of tests in Table 6.1 was determined randomly. This protected against any kind of bias in results that may have occurred from always starting tests in the same BSC.

For each test, the next several columns of Table 6.1 show the number of test coupons of each surface material (6), the number of positive-control samples (12), and the numbers of two kinds of negative control samples (4 and 4). There was one blank coupon of each surface material per test, for a total of four blank coupons. There were also four blank swabs per test. The way the test coupons were assigned to the test locations within a BSC is discussed subsequently.

The last three columns of Table 6.1 show assignments of three tasks to the two persons who performed the tests. These tasks consist of (A) sample deposition, (B) sample collection and extraction, and (C) sample analysis. Note that these descriptions are generic enough to apply to both culture and mRV-PCR analytical methods. Persons were randomly assigned to Task A, with that person also performing Task C (necessary for logistics). The other person performed Task B. The random assignment of the two persons was done in a balanced way for the originally planned 64 tests so that each person performed Tasks A/C and Task B eight times within each consecutive set of 16 tests. After the modifications to the experimental design that resulted in 68 tests instead of 64, only near balance was achieved, but this is still better than what would have been obtained solely by randomization. This near-balanced random assignment of persons to testing tasks protected against person effects (if any) possibly becoming confounded with the effects of BA surrogate, concentration, analytical method, and surface material (the test factors of interest).

Figure 6.1 displays the assignments of six test coupons for each of the four surface materials to the 24 test-coupon positions in a BSC. One-letter representations of the surface materials are used in Figure 6.1, where S = stainless steel, G = glass, V = vinyl tile, and P = plastic light cover panel. For reduced complexity, the same assignments of surface material test coupons to locations in a BSC were used for each of the pair of tests (in the two BSCs) conducted at the same time within a test run. The assignment of surface materials to BSC positions TC1 to TC24 shown in Figure 6.1 was performed to achieve balance in both rows and columns of the 2 × 12 array. Specifically, the 12 possible pairs of surface materials (S-G, S-V, S-P, G-S, G-V, G-P, V-S, V-G, V-P, P-S, P-G, P-V) occur once each in the columns of the 2 × 12 array in Figure 6.1. Also, each of the four surface materials appears three times in each row, such that there are three sets of permutations of the four materials, e.g.,

$$\{V, G, P, S\} \quad \{S, V, G, P\} \quad \{P, S, V, G\}.$$

There are many more ways to achieve these kinds of balance than the arrangement shown in Figure 6.1. In Rev. 0 of this document, Table 6.2 (which has been deleted in this Rev. 1) listed a different arrangement for each test run. However, ultimately it was decided that different arrangements

(assignments of test materials to BSC locations) for each test run would significantly complicate preparing the tests. So, the same arrangement shown in Figure 6.1 was used for every test. There should be no problem as a result of this decision, because preliminary testing showed no effect of BSC location on test results. In summary, the experimental design for the macrofoam-swab study is documented in Table 6.1 and Figure 6.1.

7.0 Gap Coverage, Advantages, and Disadvantages of this Study

Previous laboratory studies on the performance of sampling methods for surfaces contaminated by BA or surrogates have been summarized by Piepel et al. (2012). Several gaps were identified in these previous studies, including 1) lack of results quantifying FNR and RE as functions of contaminant concentration, surface material, sample collection, and other factors, 2) lack of studies to investigate and quantify the effects of sample storage and transportation conditions on the performance results of sampling and analytical methods, and 3) failure of previous studies to capture all of the sources of uncertainty affecting performance results of sampling and analytical methods under various conditions. Regarding the last gap, many of the studies investigated only short-term, within-test uncertainties (repeatability) and did not investigate run-to-run or lab-to-lab uncertainties (reproducibility). Hence, the estimated uncertainties of performance measures reported in those studies can be expected to underestimate the total uncertainty.

The purposes of the sponge-wipe study of Krauter et al. (2012) and the macrofoam-swab study discussed in this report are to generate data on FNR performance of the sponge-wipe and swab sampling and analytical methods. The resources available for the two studies were not sufficient to fill all of the gaps identified by Piepel et al. (2012). The advantages and disadvantages of the experimental design for the macrofoam-swab study presented in this report are discussed in the following bullets.

Advantages

- The macrofoam-swab study provides for developing FNR-concentration and RE-concentration equations and corresponding uncertainty expressions for combinations of BA surrogate (BAS, BG), analytical method (culture, mRV-PCR), and the four surface materials (stainless steel, glass, vinyl tile, and plastic). These equations and expressions will provide for predicting the swab FNR and RE, and their uncertainties, for any concentration within the range tested. Failure to develop results for FNR as a function of surface material, concentration, and other factors was the largest gap identified by Piepel et al. (2012).
- This study will be the first to provide for comparing the RE and FNR of macrofoam-swab sampling and analytical methods for two BA surrogates (BAS and BG). Many previous studies investigated only BG or BAS, so results of this study will provide for assessing differences in results from the two BA surrogates.
- Tests with macrofoam swabs were conducted using methods of sample collection, preparation, extraction, and culture analysis according to procedures developed (and validated for higher concentrations) by the CDC. The macrofoam-swab study investigated an mRV-PCR analytical method in addition to the culture method.
- Positive-control samples in the experimental design provide for estimating the concentration level of the BA surrogates on test coupons. The contamination on the positive-control sample associated with each pair of test coupons (as described in Section 6.2) will be used to calculate REs and will also serve as the concentration value in fitting FNR-concentration and RE-concentration equations. The positive-control sample concentrations will also allow identifying any possible variations in concentrations across test coupons within a test run because of any time trend, BSC effect, BSC position effect, and/or other effects.

- The two people who performed the (A) sample deposition, (B) sample collection and extraction, and (C) sample analytical steps of testing were assigned randomly to these tasks for each test (with the exception that the same person performed Tasks A and C). This avoided confounding personnel effects, if any, with the effects of test factors of interest (BA surrogate, concentration, and surface material).

Disadvantages

- Only the macrofoam-swab sample collection method was tested in this laboratory study. However, the sponge-wipe method was tested previously by Krauter et al. (2012).
- Preparation and extraction methods can have a significant effect on sampling performance (e.g., RE and FNR), but factors related to preparation and extraction methods were not varied in this study. Rather, the preparation and extraction methods for the culture analytical method recommended by the CDC were used. Also, an mRV-PCR analytical method was tested. Other preparation and extraction methods for culturing have been investigated and documented in the literature, and additional studies varying factors associated with those methods could be performed in the future to quantify FNRs if necessary.
- The macrofoam-swab study did not consider the effects of storage and transportation factors on swab sampling performance. Insufficient information on these factors was identified as a gap by Piepel et al. (2012). The results of a recent storage/transportation study conducted by the CDC (Perry et al. 2013) should be evaluated to decide whether additional studies are needed.
- Only one laboratory (at PNNL) was used for the testing, and therefore lab-to-lab variation was not investigated or quantified. Two lab personnel performed the steps in the 68 tests. Given the small number of personnel, their schedules, and the number of steps in the testing, it was not possible to assign personnel to capture related sources of reproducibility uncertainty. Instead, lab personnel were assigned in a randomized way to the test steps to avoid confounding any possible personnel effects with effects of the test factors (BA surrogate, concentration, and surface material).

These advantages and disadvantages should be kept in mind when 1) assessing and drawing conclusions from the results of this macrofoam-swab study and 2) planning any future laboratory studies.

8.0 Planned Data Analyses

An important part of designing an experiment is planning the future data analyses to be performed so that the experimental data will adequately support those analyses. In general, it is envisioned that the macrofoam-swab study will support completing all of the entries in Table 1 of Piepel et al. (2011), with the exception of lab-to-lab uncertainty (since only one laboratory will be used). For convenience, that table is reproduced in this report as Table 8.1.

Focusing on the most important items, the experimental design for the macrofoam-swab study presented in this report will support the following:

- estimating the FNR (and the corresponding uncertainty) for each combination of BA surrogate, concentration, surface material, and analytical method.
- estimating the surface concentrations recovered by sampling (e.g., CFU/cm^2)[(a)] and their uncertainties (e.g., %RSD) for each combination of BA surrogate, concentration, and surface material for the culture analytical method. The mRV-PCR analytical method does not provide for quantifying surface concentrations recovered by sampling.
- estimating REs (%) and their uncertainties (%RSDs) for each combination of BA surrogate, concentration, and surface material for the culture analytical method.
- fitting FNR-concentration and RE-concentration equations for each combination of BA surrogate, surface material, and analytical method. Also, assessing goodness-of-fit of the equations and quantifying the uncertainty in FNR and RE predictions made with the fitted equations.

Using results of the macrofoam-swab study, it will be possible to calculate LODs in various ways for combinations of BA surrogate, surface material, and analytical method. Different researchers define and calculate LOD in different ways, which is often inadequately discussed in journal articles and reports. The definition(s) and method(s) that will be used to calculate LODs will be discussed in future documents on the results and data analyses of the study.

Section 6 of this document describes the experimental design with a split-split-split-plot structure in which the levels of BA surrogate, the concentration, and the analytical method are subject to restrictions on randomization. The split-split-split-plot structure of the data means that there are multiple variance components for experimental testing and measurement rather than one variance component, and that subsets of the data are correlated. Special statistical data-analysis methods are required for data with such structure, and the experimental design was developed to provide for using such methods during data analyses.

- Each BA surrogate will be tested twice, so that there are four WPs (see Section 6.1 for notation and terminology). Testing the surrogates twice provides for estimating the WP uncertainty (associated with making up and testing BA surrogates at different times), although the uncertainty will be estimated with very few degrees of freedom. However, the scope and resources for the study did not allow testing each BA surrogate more than twice.

(a) CFU = colony-forming unit

- Fitting RE-concentration and FNR-concentration equations with fewer coefficients than the number of concentration levels will provide for estimating the SP uncertainty associated with concentration.
- Provided that the "analytical method" does not interact with the "BA surrogate" and/or "concentration" factors, there will be residual degrees of freedom to estimate the SSP uncertainty associated with analytical method.
- Replicate coupons of each surface material within each test will provide for estimating SSSP uncertainty.

Some data analyses (such as fitting RE-concentration and FNR-concentration equations separately for each combination of BA surrogate and analytical method) will not require the more complicated split-split-split-plot data-analysis methods because those data analyses will be performed separately. Hence, the minimal number of WP replicates will not affect those data analyses. However, if attempts are made to develop RE-concentration and FNR-concentration equations applicable to both BA surrogates and both analytical methods for a range of surface materials [e.g., by using surface roughness or other characteristic(s) as predictor variable(s)], then more complicated data-analysis methods accounting for the restrictions on randomization will be needed.

Table 8.1. Information Summarized by Piepel et al. (2011) in Their Literature Review of Previous Laboratory Studies for Sampling and Analyzing Contaminated Surfaces

Study Characteristics (in Tables 2a, 3a, 4a, and 5a)	
Reference	Author (Year) citation of publication that documents the study
Test #	A number of the form x.y where x is 1, 2, 3, … for each study, and y = 1, 2, 3, … for the tests within a study
Agent	*B. anthracis* or related surrogate
Agent deposition	Method used to deposit agent on test material
Agent concentration	Concentration or amount of agent on surface
Swab/Wipe/Vacuum type	Type or material of sample collection medium
Wetting agent	Swab or Wipe: Liquid, if any, used to wet or pre-moisten the sampling material
Vacuum technique	Vacuum: Technique in vacuuming (e.g., pattern, pressure applied)
Relative humidity	Percent relative humidity in chamber or laboratory during testing
Surface type	Material type of surface sampled
Surface area sampled	Area of surface sampled
Extraction liquid	Liquid used to extract spores from the sample
Extraction method	Method used to prepare a sample and extract the contaminant
Culture method/medium	Method/medium used to culture samples
# labs	The number of labs that participated in a study
# test runs	The number of test runs (set up and performed separately at different times)
Total # test samples	Total number of samples tested (i.e., over labs, runs, and samples within runs)
Study Results (in Tables 2b, 3b, 4b, and 5b)	
Recovery Concentration Results - Mean & %RSDs	
Reference	Same as for "Study characteristics" tables (see above)
Test #	Same as for "Study characteristics" tables (see above)
Mean (CFU/cm^2)	Mean surface concentration recovered
Lab %RSD	Lab-to-lab percent relative standard deviation, which includes the variation from preparing the samples, extraction, and analysis.
Run %RSD	Percent relative standard deviation from replicate runs of a test performed at different times.
Sample-within-run %RSD	Percent relative standard deviation from replicate tests performed at the same time (in one run)
Total %RSD	Total percent relative standard deviation (including Lab, Run, and Sample-within-run)
Recovery Efficiency (RE) – Mean & %RSDs	
RE mean (%)	Mean recovery efficiency
RE lab %RSD	Lab-to-lab percent relative standard deviation of RE
RE run %RSD	Run percent relative standard deviation of RE
RE sample-within-run %RSD	Sample-within-run percent relative standard deviation of RE
RE total %RSD	Total percent relative standard deviation of RE (including Lab, Run, and Sample-within-run)
LOD, FNR, and FPR	
Positive result	How a positive result (detection) was defined (e.g., CFU ≥ 1)
LOD definition	How the limit of detection (LOD) is defined
LOD	Value of the limit of detection
LOD SD or 95% CI (CFU/cm^2)	Standard deviation or 95% confidence interval for the LOD
FNR	False negative rate (FNR) based on controlled tests where the sampled surface was known to be contaminated but yielded a negative result
FPR	False positive rate (FPR) based on controlled tests where the sampled surface was known to be uncontaminated but yielded a positive result

9.0 Recommendations for Future Studies

As discussed previously, the swab study in this report addresses only some of the gaps in previous laboratory studies identified by Piepel et al. (2012). Hence, additional evaluations of previous studies and new experimental studies are recommended to address the following topics.

- Quantifying False Negative Rates for Vacuum Sampling Methods: Studies similar to the sponge-wipe study (Krauter et al. 2012) and the macrofoam-swab study discussed in this report should be performed for vacuum sampling and analytical methods recommended by the CDC. Other wipe, swab, or vacuum methods that are likely to be used in BA contamination events should also be studied (see the discussion in the following bullet). Piepel et al. (2012) summarized literature results from five vacuum studies (noting that more were needed). However, none of these studies investigated a sufficient number of concentrations at low enough concentration levels to quantify the FNR as a function of concentration and any other affecting variables. Quantifying the FNR is critical to being able to calculate the confidence in detection and clearance decisions when all samples yield negative results.
- Quantifying Performance of Different Methods: The CDC has performed validation studies for a sponge-wipe method, a macrofoam-swab method, and corresponding recommended extraction and analytical methods. A similar study for CDC-validated vacuum sampling, extraction, and analytical methods is planned. However, it is reasonable to expect that biological contamination events in various parts of the United States may result in wipe, swab, and vacuum sample collection methods that differ from methods recommended by the CDC, as well as different storage/transportation, extraction, and analytical methods. Previous studies summarized by Piepel et al. (2012) showed that different sample collection methods and different extraction methods can have significantly different performance results. Hence, it would be prudent to quantify the FNR performance of the two or three most-likely-to-be-used swab, wipe, and vacuum sample collection methods, transportation/storage methods, extraction methods, and analytical methods. Therefore, it is recommended that 1) the two or three of the most-likely-to-be-used methods of each type be identified and 2) the existing literature data summarized by Piepel et al. (2012) be evaluated to see how well the performance of the most-likely methods has been investigated. If there are gaps, then additional experimental studies may be needed.
- Reproducibility Uncertainty: The majority of previous laboratory studies only quantified within-laboratory uncertainty (Piepel et al. 2012) and sometimes only short-term, within-laboratory uncertainty. Lab-to-lab uncertainty and other sources of reproducibility uncertainty (e.g., test personnel, instruments, etc.) need to be quantified as functions of any affecting variables (e.g., contaminant concentration). It was not possible to include these features in the sponge-wipe study (Krauter et al. 2012) or in the macrofoam-swab study discussed in this report. Hence, it is recommended that reproducibility uncertainty be quantified in future experimental studies for the sponge-wipe and macrofoam-swab methods as well as in any future vacuum studies.
- Transportation and Storage Effects: The Almeida et al. (2008) study and the recent CDC study of storage/transportation effects on sample results (Perry et al. 2013) should be evaluated to determine whether additional studies are needed to quantify storage and transportation effects as functions of any affecting variables (e.g., contaminant concentration).

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