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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

April 2014

Pacific Northwest
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Pacific Northwest National Laboratory
Richland, Washington 99352

Executive Summary

This report describes 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 for low concentrations of *Bacillus anthracis* Sterne (BAS) and *Bacillus atrophaeus* Nakamura (BG) spores on several surface materials. The experimental design was developed at Pacific Northwest National Laboratory (PNNL), where the testing will be 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 available information. 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 is 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 for examining the performance of the macrofoam swab method with two BA surrogates (BAS and BG) at eight low concentrations on four surface materials. This report also discusses the planned results and data analyses. The key response variables include 1) contamination on test coupons of the surface materials (obtained from positive-control samples), 2) contamination recovered from test coupons by swab sampling, 3) RE, and 4) FNR. These response variables will be measured by plating/counting and polymerase chain reaction (PCR).

Ideally, the swab study would use dry aerosol deposition of the contaminant 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 low enough concentrations on test coupons 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 enough concentrations of the contaminant on test coupons that false negatives were achieved. For this reason, the swab study will be conducted in a laboratory using a similar liquid deposition method as described by Krauter et al. (2012).

The experimental design for the swab laboratory study will 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 will be 2 inches by 2 inches. There will be two sets of eight test runs for each of the two BA surrogates, for a total of 32 test runs. During each test run, both the plating/counting and PCR analytical methods will be used. Hence, here will be 64 tests total. As a practical matter for performing the tests, the eight test runs per surrogate will be conducted as a set, with four sets of eight test runs. The eight surface concentrations of a BA surrogate will be randomly assigned to the eight test runs for that surrogate. It is expected that some of the very low concentrations may present challenges for deposition, sampling, and analysis. However, such tests are needed to obtain FNRs ranging up (or near) to one.

The experimental design for this laboratory study is a split-split-split-plot experiment (Steel and Torrie 1960, Chapter 12; Myers and Montgomery 1995, Section 9.6) in which “BA surrogate” is the whole-plot (WP) factor, “concentration” is the sub-plot (SP) factor, and “analytical method” (plating/counting and PCR) is the sub-sub-plot (SSP) factor. Over the two sets of eight test runs for a BA surrogate, there will be 12 test coupons for each of the four surface materials. This will provide a good basis for calculating empirical FNR values (i.e., FNR = x/12, where x = number of false negatives) for each surface material. Positive-control (reference) samples will be generated by placing the same number of drops of BA surrogate solution on a growth plate (for plating/culture) or in a collection tube (for PCR) as will be placed on associated test coupons. Creating reference samples at the same time as test coupons provides 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 estimates of actual contaminant concentrations will be used during the data analysis phase 1) to calculate RE values for the swab sampling and analytical method and 2) as the concentrations for fitting RE- and FNR-concentration equations.

Data analyses for the swab laboratory study will support 1) estimating the FNR for each combination of BA surrogate, concentration, and surface material, 2) estimating the surface concentrations (e.g., CFU/cm^2) and their uncertainties (e.g., standard deviation [SD] or percent relative standard deviation [%RSD]) for each combination of BA surrogate, concentration, and surface material, 3) estimating REs (%) and their uncertainties (SD or %RSD) for each combination of BA surrogate, concentration, and surface material, 4) fitting FNR-concentration and RE-concentration equations for each combination of BA surrogate, surface material, and analytical method, 5) assessing goodness-of-fit of the equations, and 6) quantifying the uncertainty in FNR and RE predictions made with the fitted equations. 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 limit of detection 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 (i) the two BA surrogates, and (ii) the two analytical methods.

In summary, the data resulting from the 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
DoD	U.S. Department of Defense
DOE	U.S. Department of Energy
EPA	U.S. Environmental Protection Agency
FBI	Federal Bureau of Investigation
FNR	false negative rate
FPR	false positive rate
GAO	Government Accountability Office
LOD	limit of detection
NIST	National Institute of Standards and Technology
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNNL	Pacific Northwest National Laboratory
QC	quality control
RE	recovery efficiency
SD	standard deviation
S&T	Science and Technology Directorate
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

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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 is 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 BA or surrogates. The experimental design in this report was developed at Pacific Northwest National Laboratory (PNNL) and the laboratory study will be performed by PNNL.

The laboratory study will collect data to characterize the performance of swab sample collection, extraction, and analysis by plating/culture procedures reported by the Centers for Disease Control and Prevention (CDC) (Hodges et al. 2010). The swab sampling method is also recommended in the Validated Sampling Plan Working Group (VSPWG) sampling reference guide.[a] Additionally, the swab study will evaluate a polymerase chain reaction (PCR) method for analysis of swab samples using a modification of the protocol reported by Létant et al. (2011).

The swab study will investigate the effects of selected independent variables on several response (dependent) variables. For several reasons, it was necessary to limit the number of independent variables investigated in the swab study. The four independent variables that will be varied in the study are the 1) *Bacillus anthracis* surrogate (BAS, BG), 2) surface concentration of the surrogate, 3) surface material, and 4) analytical method (plating/counting, 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) will be investigated. The eight concentrations may be different for samples analyzed by plating/counting versus by PCR if scoping tests show these methods may have significantly different FNR performances. The variables that will be held constant or varied in the swab study are discussed further in Sections 2 and 3, respectively. The response variables for which data will be collected include the 1) contamination on positive-control samples associated with test coupons, 2) contamination recovered from test coupons by swab samples, 3) RE, and 4) FNR.

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

Concentrations of BA necessary to cause illness or symptoms may include values low enough that the FNR > 0. Hence, estimates of FNR, RE, and other quantities (e.g., limits of detection) 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 results after a contamination event. The swab study will generate data to support developing RE and FNR equations for the two BA surrogates as functions of

(a) *Reference Guide for Developing and Executing Bacillus anthracis Sampling Plans in Indoor Settings* (Draft, pre-decisional), Validated Sampling Plan Working Group, 2012.

contaminant surface concentration for combinations of the four surface materials and two analytical methods. These RE-concentration and 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 (S&T) and includes experts from the CDC, the U.S. Environmental Protection Agency (EPA), the U.S. Department of Defense (DoD), and the Federal Bureau of Investigation (FBI). Although not formally members of the VSPWG, staff from the National Institute of Standards and Technology (NIST) 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 swab study conducted using the experimental design in this report will contribute to addressing both of the GAO's main concerns. Specifically, for swab samples collected, extracted, and analyzed using the selected methods, the study will yield equations relating the FNR to the surface concentration of a BA surrogate for combinations of BA surrogate, surface material, and analytical

(a) *Reference Guide for Developing and Executing Bacillus anthracis Sampling Plans in Indoor Settings* (Draft, pre-decisional), Validated Sampling Plan Working Group, 2012.

method. The four surface materials that will be tested in the swab study are expected to span a range of surface roughness values 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 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 types sampled by a macrofoam swab and analyzed by one of two analytical methods. The CDC-validated method for swab sampling and extraction will be used with the CDC-validated plating/culture analytical method and with a 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 concentration of the contaminant, the surface material being sampled, and the 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 swab study discussed in this report was partly motivated by a review of the literature on previous laboratory studies by PNNL (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 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 PCR analysis (which is not part of CDC methods).

1.3 Staff and Funding

The work 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 specific contributors are listed as authors of this report.

The work at PNNL was funded by the Chemical and Biological Research and Development Branch of the Chemical and Biological Division of DHS. The work was funded 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 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 samples (i.e., positive and negative controls) included in the swab study are discussed in Section 4. Preliminary tests that will be 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 to be Held Constant in the Swab Study

Table 2.1 lists the factors that will be held constant during the swab study.

Table 2.1. Factors to be Held Constant in the Swab Study

Factor	Test Level
Sample collection media	Macrofoam swab[(a)]
Sample/Coupon Size	2-inch by 2-inch (25.806 cm^2)
Wetting agent	PBS-T (PBS with 0.02% Tween 80)[(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 results report.
(b) PBS = phosphate buffered saline; PBS-T = PBS 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 is not sufficient to use different laboratories as part of the study.

The macrofoam swab that will be used for the study is described by Hodges et al. (2010). Test coupons that are 2 inches by 2 inches will be used. A liquid deposition method similar to that used by Krauter et al. (2012) will be used to deposit spores on test coupons and positive-control samples. This liquid deposition method can reliably achieve lower concentrations of the contaminants on test coupons, so that adequate numbers false negatives can 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 comprise a very large study and be beyond the scope of this effort. Hence, it is envisioned that these factors could be investigated in future laboratory studies if deemed necessary.

3.0 Factors to be Varied in the Swab Study

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

Two BA surrogates will be tested: *Bacillus atrophaeus* Nakamura (BG), and *Bacillus anthracis* Sterne (BAS). Based on the literature review of Piepel et al. (2012), it appears that BG and BAS have not both been previously tested in the same study. Testing both in this study will provide 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 will be 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 swab (many of which are very smooth). Spanning the range of surface roughness values will allow applying the results of the swab study to various surface materials that might be sampled by a swab.

Table 3.1. Factors that Will Be Varied in the Swab Study

Factor	Test Levels
BA surrogates	*Bacillus atrophaeus* Nakamura (ATCC 9372), *Bacillus anthracis* Sterne
Surface materials	Stainless steel, glass, vinyl tile, and plastic (from a ceiling light cover panel)
Surrogate surface concentrations	Eight 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	Plating/culture, PCR
Biosafety cabinets[b]	Two Nuaire Class II, Type A2 6-foot biosafety cabinets (BSCs) will be used to enable faster progress in testing.
Personnel for sample collection, processing, and analytical[b]	Two to three personnel per test run to perform the sample collection, extraction, and analytical steps of the work

(a) The eight concentrations may be different for combinations of the two BA surrogates (BAS, BG) and the two analytical methods (plating, PCR). The set(s) of eight concentrations will be determined by preliminary, range-finding scoping tests conducted prior to the actual test matrix (see Section 5).

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

Eight different surface concentration levels will be chosen to span the range of FNR values (0 to 1) for the four surface materials. Because FNR performance of the swab sampling and analytical methods may be different for the BAS and BG surrogates, and/or for the culture and PCR analytical methods, it may be necessary to choose different sets of eight concentrations for combinations of these two factors. The process for selecting the eight concentration levels to use in tests with each BA surrogate and each analytical method is discussed in Section 5.1. The number of concentrations (eight) 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 still be four concentrations to span the interior of the 0 to 1 range for FNR.

There will be 12 test coupons for each surface material for each combination of BA surrogate, concentration, and analytical method. Having 12 coupons for each surface material will provide 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 will be used to produce test results: plating/culture and PCR. In the previous sponge-wipe study (Krauter et al. 2012), only the plating/culture method was used. It was decided to also include the PCR method in this swab study because of only incremental additional cost to do so.

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

- To permit completion of testing in a reasonable time, two BSCs will be used. The combinations of settings of the test factors of interest will be assigned to BSCs in a balanced way so that any possible difference between the two BSCs will not be confounded with the effects of the test factors of interest. Also, preliminary tests discussed in Section 5.2 will be performed to assess whether there is any difference in test results using the two BSCs.
- Two persons will perform the sample collection, extraction, and analytical (i.e., plating/counting or PCR) steps of the sampling and analytical work. A third person will be available as a backup. The personnel performing each task in the process will be 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 are varied as part of the experimental design.

4.0 Quality Control Samples in the Swab Study

Each test run in the swab study will include a complement of quality control (QC) samples, which will be thoroughly documented in the PNNL test procedures. The QC samples comprise positive and negative control samples.

All test coupons within a test run will nominally have the same concentration of BA surrogate, which will be achieved with a specified number of drops of the BA surrogate solution. Hence, positive controls will receive the same number of drops of the solution as test coupons. The number of drops for positive controls will be placed

- on a growth media plate for analysis by plating/counting
- in a collection tube for DNA extraction and 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 BA surrogate, eight concentrations, four surface materials, and two analytical methods (plating/counting, PCR). Note that the PCR results will involve relative quantification.

The negative controls will consist of 1) blank coupons that will be processed the same as spore-seeded coupons and 2) blank swabs (process negatives) that will be 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 will be documented because they provide for estimating the false positive rate (FPR) of the swab method.

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

5.0 Preliminary Tests

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

5.1 Preliminary Tests to Determine Contaminant Concentrations

Preliminary “range finding” tests will be performed before the actual tests in the swab study to determine the eight surface concentration levels that will be used for testing. If necessary, different sets of the eight concentrations will be developed for the combinations of the two BA surrogates (BAS, BG) and the two analytical methods (plating/culture, PCR). A given set of eight concentrations of the BA surrogates (BAS, BG) will be 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 FNR values spanning the range of 0 to 1. The data from the test runs will be used to fit an FNR-concentration curve and RE-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 changed as needed.

The set of eight concentrations will be chosen with the expectation that 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 $0 < \text{FNR} < 1$. 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 that will be tested in the swab study will be chosen based on preliminary/scoping tests conducted before the actual tests. If necessary, separate sets of eight concentrations will be chosen for tests with combinations of the two BA surrogates (BAS, BG) and the two analytical methods (plating/counting, PCR).

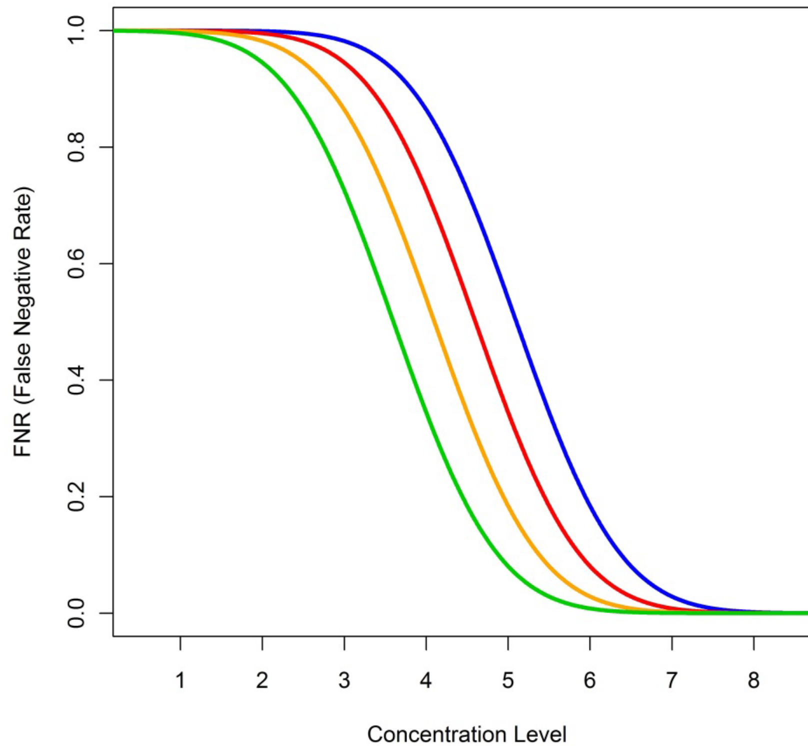

Figure 5.1. An Example of Eight Concentration Levels Selected to Span the Range of FNR Values for Four Surface Materials. The four curves represent hypothetical relationships between FNR and concentration.

5.2 Preliminary Tests to Assess Other Experimental Variables

Preliminary tests will also be conducted prior to the actual tests to assess the following experimental factors that may impact the experimental design.

- Two Nuaire Class II, Type A2 BSCs (which are adjacent in the same laboratory) will be used for testing. Preliminary tests will assess whether there is any systematic difference in results from the two BSCs.
- Tests will be conducted with test coupons and positive-control samples at various test locations within the planned work area of a BSC. Preliminary tests will be 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 and 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 will be conducted to assess this assumption.

The experimental design of the swab study discussed in Section 6 assumes that there will be 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. If the preliminary testing indicates these assumptions are wrong, revisions to the experimental design presented in Section 6 may be needed.

5.3 Preliminary Tests to Optimize PCR Detection

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

- Preliminary tests will be conducted to determine the most efficient DNA extraction protocol. 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 will determine the methods for optimal DNA recovery for low concentrations of spores.
- Preliminary tests using published PCR primers and probes will be conducted to down-select the primer probe set that is 1) robust and 2) has a low limit of detection.

The preliminary tests to optimize PCR conditions will elucidate the methods for 1) DNA extraction and 2) PCR detection. Results of these tests will be provided in the PNNL procedures for the experimental work.

6.0 Experimental Design for the Swab Study

This section discusses the experimental design of the swab study. Section 6.1 discusses the restrictions on randomization that will apply in conducting the swab study and introduces the statistical terminology for experiments with restrictions on randomization. Section 6.2 discusses how the tests will be 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 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 all tests. In such cases, one or more restrictions on randomization may occur. For the swab study, there are three restrictions on randomization:

- It would be too complicated and would risk cross-contamination to randomly intermix tests with the two BA surrogates. Tests with a given BA surrogate will be performed as four sets of 16 tests, with each BA surrogate tested twice.
- After a given concentration of a BA surrogate is prepared, all tests with that concentration will be performed as a set.
- Finally, it will simplify matters to conduct together in subsets the tests with a given combination of BA surrogate and concentration to be analyzed by plating/counting and by PCR.

For each combination of BA surrogate, concentration, and analytical method, all 12 replicate test coupons of each of the four surface materials will be 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; Myers and Montgomery 1995, Section 9.6). 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" (plating/counting and 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 (Myers and Montgomery 1995).

(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[a] will be assigned randomly to the eight test runs for each of the two analytical methods (plating/counting, PCR) to protect against confounding any possible time-trend effects with concentration effects over the eight test runs. Over all tests for a given BA surrogate, the four nonporous surface materials will be investigated with 12 test coupons for each material. Having 12 test coupons per material will provide a good basis for calculating empirical FNR values (i.e., FNR = x/12, where x = the number of false negatives ranging from 0 to 12) for each surface material.

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 will be 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 will be used for consistency of testing both BA surrogates.

The numbers and placements of test coupons, positive-control (reference) samples, and negative control samples within the BSCs are an important part of the experimental design for the 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, concentration, surface material, and analytical method. Section 4 describes how positive-control samples will be generated for both the plating/counting and 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 are 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, and there is airflow through the BSCs that has been observed to cause faster drying of liquid depositions on samples at the back of a BSC than at the front. It was judged ideal to have two rows of 12 locations within a BSC for test coupons and positive-control samples, with the two rows located in the middle (front to back) of a BSC to avoid having samples located near the back of the 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 require 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.

(a) It is assumed in this discussion that the eight concentrations will be the same for the two analysis methods (plating/counting, PCR). If not, then there will be more than eight concentrations total over the two analysis methods, depending on the number of concentrations that overlap. This will lead to there being more than 16 total test runs for a given BA surrogate.

To reduce the number of BSC runs, it was decided to use an arrangement of three rows of 12 locations for test coupons and positive-control samples within a BSC. The second (middle) of the three rows would contain positive-control samples, while the other two rows would contain test coupons (see Figure 6.1). Hence, it would only take two BSC runs (each containing 24 test coupons and 12 positive controls) 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 is 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 will be associated with the two test coupons on either side of it in the first and third rows. Provided that the preliminary tests discussed in the second bullet of Section 5.2 show that there are no location effects among the three rows of 12 samples in a BSC, it will not be necessary to have a one-to-one relationship between positive-control samples and test coupons. Four coupon negative controls will be located within a BSC adjacent to the needed consumables (tips) and equipment (trash, vortex, pippetor, and stir plate). Coupons will be placed in sterile petri dishes to allow for ease of set up.

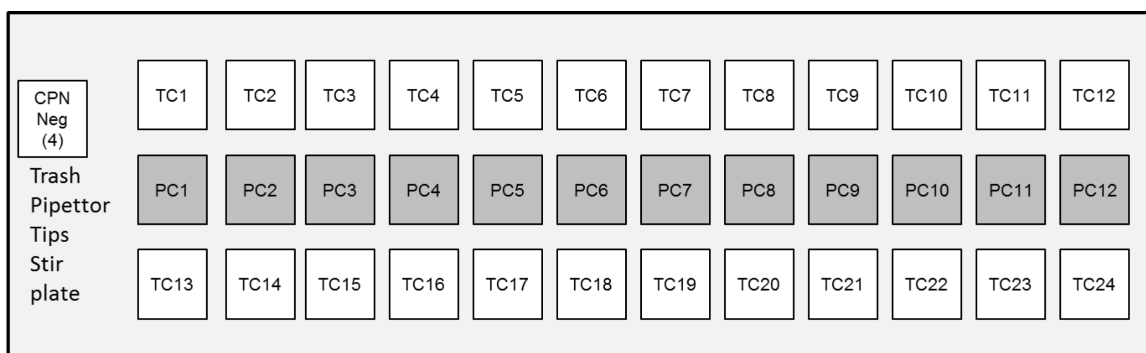

Figure 6.1. BSC Testing Configuration Showing the Locations of the 24 Test Coupons (TC1 – TC24; white fill), the 12 Positive Controls (PC1 – PC12; gray fill), and the Four Negative Coupon Controls (CPN Neg (4); white fill) for a Given Test Run and BSC. Additionally the needed consumables and equipment will be located to the left of the test coupons (trash, pipettor, tips, and stir plate). 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 Swab Study

Table 6.1 lists the general aspects of the split-split-split-plot experimental design for the swab study. The first column lists the *test run* numbers, 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. Each BA surrogate will be tested twice, with eight test runs (concentrations) each time, so there are a total of $4 \times 8 = 32$ test runs. With two tests per test run, there are 64 tests, each of which corresponds to a combination of BA surrogate, concentration, and analytical method. The second column of Table 6.1 lists the test numbers 1–64, 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 surrogates to be tested, where there are four sets of 16 test runs of the BA surrogates in the order BAS, BG, BAS, BG. The 12 coupons of each surface material for

a given combination of BA surrogate, nominal concentration, and analytical method were partitioned with six coupons appearing in each of the two sets of eight test runs (16 tests) with a given surrogate. Testing the surrogates twice each in sets of eight test runs (16 tests) instead of once each in sets of 16 test runs (32 tests) is 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 will be tested in two different sets of 16 tests, the working spore stock for each nominal concentration will be made twice. The actual concentrations of working spore stock may not be exactly the same as the nominal values in the two sets of 16 tests for a BA surrogate, but that is acceptable and is what provides for statistically comparing the results for the BA surrogates. Only testing each surrogate twice will provide a very limited basis for statistically comparing test results for the two surrogates. However, the budget for testing did not allow for testing the surrogates more than twice each.

The fourth column of Table 6.1 lists the nominal concentration for each test. The eight nominal concentrations (denoted C1–C8 where C1 is the lowest concentration and C8 is the highest concentration) 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 to provide for obtaining the desired FNR data (as discussed in Section 5.1). The pseudo-random assignment protects against any kind of trends in results that may occur over time, so that such effects are not confounded with the effect of concentration on the response variables.

The fifth column of Table 6.1 identifies which of the two analytical methods (plating/culture, PCR) will be used for each test. All tests in one of the BSCs will be analyzed with one method, while all of the tests in the other BSC will be analyzed with the other method.

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

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 will be one blank coupon of each surface material per test, for a total of four blank coupons. There will also be four blank swabs per test. How 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 will perform 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 plating/culture and PCR analytical methods. Persons were randomly assigned to Task A, with that person also performing Task C (necessary for logistics). The other person performs Task B. The random assignment of the two persons was done in a balanced way so that each person performs Tasks A/C and Task B eight times within each consecutive set of 16 tests. This balanced random assignment of persons to testing tasks protects 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).

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

Test Run	Test[b]	BA Surrogate[c]	Concen-tration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons: Stainless Steel	Glass	Vinyl Tile	Plastic	# Positive Controls[g]	# Negative Controls: Coupons[h]	Swabs[i]	Testing Task[j]: A	B	C
1	1	BAS	C5	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
	2	BAS	C5	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
2	3	BAS	C6	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
	4	BAS	C6	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
3	5	BAS	C1	PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	6	BAS	C1	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
4	7	BAS	C3	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	8	BAS	C3	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
5	9	BAS	C2	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
	10	BAS	C2	PCR	BSC2	6	6	6	6	12	4	4	1	2	1
6	11	BAS	C7	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	12	BAS	C7	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
7	13	BAS	C4	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	14	BAS	C4	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
8	15	BAS	C8	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	16	BAS	C8	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
9	17	BG	C5	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
	18	BG	C5	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
10	19	BG	C6	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
	20	BG	C6	PCR	BSC2	6	6	6	6	12	4	4	1	2	1
11	21	BG	C1	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	22	BG	C1	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
12	23	BG	C3	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
	24	BG	C3	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
13	25	BG	C2	PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	26	BG	C2	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
14	27	BG	C7	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	28	BG	C7	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
15	29	BG	C4	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
	30	BG	C4	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 Swab Study (contd)

Test Run	Test[b]	BA Surrogate[c]	Concen-tration[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
16	31	BG	C8	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
	32	BG	C8	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
17	33	BAS	C5	PCR	BSC2	6	6	6	6	12	4	4	1	2	1
	34	BAS	C5	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
18	35	BAS	C6	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	36	BAS	C6	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
19	37	BAS	C1	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	38	BAS	C1	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
20	39	BAS	C3	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
	40	BAS	C3	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
21	41	BAS	C2	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
	42	BAS	C2	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
22	43	BAS	C7	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
	44	BAS	C7	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
23	45	BAS	C4	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
	46	BAS	C4	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
24	47	BAS	C8	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
	48	BAS	C8	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
25	49	BG	C5	P/C	BSC2	6	6	6	6	12	4	4	2	1	2
	50	BG	C5	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
26	51	BG	C6	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
	52	BG	C6	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
27	53	BG	C1	PCR	BSC2	6	6	6	6	12	4	4	2	1	2
	54	BG	C1	P/C	BSC1	6	6	6	6	12	4	4	1	2	1
28	55	BG	C3	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	56	BG	C3	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
29	57	BG	C2	P/C	BSC1	6	6	6	6	12	4	4	2	1	2
	58	BG	C2	PCR	BSC2	6	6	6	6	12	4	4	1	2	1
30	59	BG	C7	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
	60	BG	C7	PCR	BSC1	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 Swab Study (contd)

Test Run	Test[b]	BA Surrogate[c]	Concen-tration[d] # Spores	Analytical Method[e]	Biosafety Cabinet[f]	# Test Coupons: Stainless Steel	Glass	Vinyl Tile	Plastic	# Positive Controls[g]	# Negative Controls: Coupons[h]	Swabs[i]	Testing Task[j]: A	B	C
31	61	BG	C4	PCR	BSC1	6	6	6	6	12	4	4	2	1	2
	62	BG	C4	P/C	BSC2	6	6	6	6	12	4	4	1	2	1
32	63	BG	C8	PCR	BSC1	6	6	6	6	12	4	4	1	2	1
	64	BG	C8	P/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 Table 6.2.
(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 numbers of spores/coupon or control sample, where the coupons are 2 in. × 2 in. (25.806 cm^2). The concentrations (C1–C8, with C1 being the lowest concentration and C8 being the highest concentration) were assigned to test runs in a pseudo-random manner.
(e) P/C = plating/culture, PCR = polymerase chain reaction. The two analytical methods were randomly assigned to BSC1 and BSC2 for each pair of test runs at the same concentration, such that each analytical method is used eight times within the sets of Test Runs 1–16, 17–32, 33–48, and 49–64.
(f) Two BSCs are located adjacent to each other on one wall of a laboratory. The left one is designated BSC1 and the right one is designated BSC2. The two BSCs will run tests simultaneously, with the one to be started first in each pair of tests determined randomly.
(g) There will be one positive-control (reference) sample associated with the two test coupons on either side of it as shown in Figure 6.1.
(h) For each test run with a BSC, there will be one blank (uncontaminated) coupon of each of the four surface materials, for a total of four negative coupon controls.
(i) For each test run with a BSC, there will be 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 will perform the testing tasks. These tasks are: (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 run. For logistics reasons, the person who performs Task A will also perform Task C. Persons 1 and 2 were randomly assigned to Tasks A and B for each test run, such that the each person performs Task and B eight times within the sets of Test Runs 1–16, 17–32, 33–48, and 49–64. The person performing Task C was set to be the same as the person assigned to Task A.

Table 6.2 lists for all 64 tests the assignments of six test coupons for each of the four surface materials to the 24 test-coupon positions in a BSC (shown in Figure 6.1). One-letter representations of the surface materials are used in Table 6.2, 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, as shown in Table 6.2.

The assignment of surface materials to BSC positions TC1 to TC24 shown in Table 6.2 was performed to achieve balance in both rows and columns of the 2 x 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 x 12 array in Figure 6.1. Also, the four surface materials appear three times each in each row, such that there are permutations of the four materials within each of three sets, e.g.,

{V, G, P, S}   {S, V, G, P}   {P, S, V, G}.

An example of the 2 x 12 arrangement of surface materials with both row and column balance as described above is given by

G	S	V	P	V	G	P	S	S	P	G	V
PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
S	P	G	V	P	V	S	G	V	G	P	S

where the middle row of positive-control samples is shown as in Figure 6.1. There are more possible ways to achieve row and column balance than the 32 test runs (64 tests in pairs) shown in Table 6.2, so the assignments of surface materials to test coupon positions in a BSC were made so that each of the 32 rows in Table 6.2 are unique.

Row and column balance of this kind should not be necessary if the preliminary testing shows there are no location effects on the work area of a BSC. However, such balance protects against location effects occurring during actual testing when they did not occur during preliminary testing. Otherwise, such location effects could be confounded with effects of surface materials, which would be undesirable.

In summary, the experimental design for the swab study is documented in Table 6.1, Table 6.2, and Figure 6.1.

Table 6.2. Assignment of Surface Material Test Coupons[(a)] to Biosafety Cabinet Positions

Test Run	Tests	BSC Test Coupon Position[(b)]																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1, 2	P	S	V	G	V	P	S	G	G	V	S	P	V	G	S	P	G	S	P	V	S	P	V	G
2	3, 4	G	V	S	P	S	V	P	G	S	V	G	P	V	P	G	S	P	G	V	S	V	S	P	G
3	5, 6	V	G	P	S	V	G	S	P	P	S	V	G	P	S	G	V	G	V	P	S	V	G	S	P
4	7, 8	S	P	G	V	P	G	V	S	S	P	V	G	G	V	P	S	G	S	P	V	P	S	G	V
5	9, 10	G	P	V	S	S	G	V	P	G	S	P	V	V	S	G	P	V	S	P	G	P	G	V	S
6	11, 12	S	V	P	G	G	V	S	P	G	P	S	V	G	S	V	P	S	P	V	G	V	S	P	G
7	13, 14	P	G	V	S	V	P	S	G	G	V	S	P	S	V	P	G	S	G	V	P	S	G	P	V
8	15, 16	G	V	S	P	P	V	S	G	V	S	G	P	S	G	P	V	G	S	V	P	P	G	V	S
9	17, 18	V	S	P	G	P	S	G	V	G	V	P	S	P	G	V	S	G	V	P	S	V	G	S	P
10	19, 20	G	S	P	V	G	P	V	S	S	G	V	P	P	G	V	S	V	S	G	P	V	S	P	G
11	21, 22	S	P	V	G	S	G	V	P	V	G	P	S	G	V	S	P	P	V	G	S	P	S	G	V
12	23, 24	P	G	S	V	S	V	G	P	V	P	G	S	S	V	G	P	P	G	S	V	S	G	P	V
13	25, 26	V	S	P	G	G	P	S	V	S	P	V	G	G	P	V	S	V	S	G	P	V	G	S	P
14	27, 28	P	S	V	G	P	V	S	G	G	V	P	S	S	V	G	P	G	S	P	V	S	P	V	G
15	29, 30	G	P	S	V	S	V	P	G	S	P	G	V	S	V	G	P	P	G	S	V	V	G	P	S
16	31, 32	P	S	V	G	V	G	P	S	V	S	G	P	V	G	P	S	G	P	S	V	S	P	V	G
17	33, 34	S	G	V	P	S	V	G	P	S	P	G	V	P	V	G	S	V	P	S	G	G	V	P	S
18	35, 36	G	P	V	S	P	G	V	S	S	G	P	V	S	V	G	P	S	V	P	G	V	P	G	S
19	37, 38	P	V	G	S	S	P	G	V	G	S	V	P	S	G	V	P	V	G	P	S	S	G	P	V
20	39, 40	S	P	V	G	V	G	S	P	V	G	P	S	P	S	G	V	P	S	V	G	S	P	V	G
21	41, 42	S	G	P	V	V	S	G	P	S	V	P	G	G	P	V	S	P	V	S	G	P	G	S	V
22	43, 44	V	G	S	P	V	S	P	G	S	P	G	V	G	S	P	V	P	G	S	V	V	G	P	S
23	45, 46	G	P	V	S	P	S	V	G	G	S	P	V	V	S	P	G	G	V	S	P	S	P	V	G
24	47, 48	S	G	P	V	P	V	S	G	G	V	P	S	P	V	S	G	G	P	V	S	P	S	V	G
25	49, 50	P	S	V	G	V	G	S	P	V	P	S	G	V	G	S	P	G	V	P	S	P	G	V	S
26	51, 52	P	S	V	G	S	P	V	G	S	G	V	P	S	G	P	V	V	G	S	P	P	S	G	V
27	53, 54	V	G	S	P	S	V	G	P	G	S	V	P	S	P	V	G	P	G	V	S	S	G	P	V
28	55, 56	G	S	V	P	S	V	P	G	G	P	V	S	P	V	S	G	P	G	V	S	V	S	P	G
29	57, 58	V	G	P	S	V	S	G	P	S	G	P	V	G	S	V	P	P	G	V	S	V	P	G	S
30	59, 60	S	G	P	V	S	G	V	P	V	P	S	G	V	S	G	P	G	P	S	V	G	S	P	V
31	61, 62	G	V	S	P	S	V	G	P	P	V	G	S	P	G	V	S	P	S	V	G	V	P	S	G
32	63, 64	G	P	S	V	S	V	G	P	P	V	G	S	V	S	P	G	G	S	P	V	G	P	S	V

(a) S = stainless steel, G = glass, V = vinyl tile, P = plastic.
(b) The position numbers of test coupons in a BSC are shown in 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 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 swab-study experimental design presented in this report are discussed in the following bullets.

<u>Advantages</u>

- The swab study provides for developing FNR-concentration and RE-concentration equations and corresponding uncertainty expressions for combinations of BA surrogate (BAS, BG), analytical method (plating/counting, 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 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 will be conducted using swab sample collection, preparation, extraction, and plating/culture analytical methods according to procedures developed (and validated for higher concentrations) by the CDC. In addition, the swab study will investigate a PCR analytical method in addition to the plating/culture method.
- Positive-control (reference) samples will 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 be used to identify 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.

- Two people who will perform 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 performs Tasks A and C). This will avoid confounding personnel effects, if any, with the effects of test factors of interest (BA surrogate, surface material and concentration).

Disadvantages

- Only the swab sample collection method will be 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 will not be varied in this study. Rather, the preparation and extraction methods for the plating/counting analytical method recommended by the CDC will be used. Also, a PCR analytical method will be tested. Other preparation and extraction methods for plating/counting have been investigated and documented in the literature, and additional studies varying factors associated with those methods could be performed in the future if necessary.
- The swab study will 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 should be evaluated to decide whether additional studies are needed.
- Only one laboratory (at PNNL) will be used, and therefore lab-to-lab variation will not be investigated or quantified. Two lab personnel will perform the steps in the 64 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 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 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 swab study presented in this report will support:

- Estimating the FNR and the corresponding uncertainty for each combination of BA surrogate, concentration, surface material, and analytical method.
- Estimating the surface concentrations (e.g., CFU/cm^2) and their uncertainties (e.g., %RSD) for each combination of BA surrogate, concentration, surface material, and analytical method.
- Estimating REs (%) and their uncertainties (%RSDs) for each combination of BA surrogate, concentration, surface material, and 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 swab study, it will be possible to calculate various kinds of "limits of detection" for combinations of BA surrogate, surface material, and analytical method. The *limit of detection* (LOD) is controversial because different researchers define and calculate it in different ways, which is often inadequately discussed. The definitions and methods that will be used to calculate LODs will be discussed in a separate document 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, concentration, and 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, 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 such methods.

- Each BA surrogate will be tested twice, so that there are four WPs (see Section 6.1 for notation and terminology). Such WP replicates provide for estimating the WP uncertainty (associated with making up and testing BA surrogates at different times), although the uncertainty will be estimated with very small degrees of freedom. However, the scope and resources for the study did not allow testing each BA surrogate more than twice.
- Fitting 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 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 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 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 swab study discussed in this report. Hence, it is recommended that reproducibility uncertainty be quantified in future experimental studies for the sponge-wipe and 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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