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United States
Environmental Protection
Agency

Test/QA Plan for Systematic Investigation of Fumigant Technologies for Decontamination of Biological Agents from Contaminated Building Materials

Office of Research and Development
National Homeland Security Research Center

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TEST/QA PLAN FOR

Systematic Investigation of Fumigant Technologies for Decontamination of Biological Agents from Contaminated Building Materials

Prepared under
GSA Contract Number GS-23F-001L-3
Task Order Number 1123
EPA Task Order Project Officer
Shawn P. Ryan

Prepared by
Battelle
Columbus, Ohio

Office of Research and Development
National Homeland Security Research Center, Decontamination and Consequence Management Division

A
Project Management

A1 TITLE AND APPROVAL PAGE

EPA/Battelle Approval of Test/QA Plan

For

Systematic Investigation of Fumigant Technologies for
Decontamination of Biological Agents from Contaminated Building Materials

Shawn P. Ryan — June 13, 2007
Shawn P. Ryan, Ph.D. — **Date**
EPA Task Order Project Officer

Eletha Brady-Roberts — May 31, 2007
Eletha Brady-Roberts — **Date**
NHSRC Quality Assurance Manager

Karen Riggs — 6/14/07
Karen Riggs — **Date**
Battelle TTEP Manager

Zachary Willenberg — 6-14-07
Zachary Willenberg — **Date**
Battelle Quality Assurance Manager

BATTELLE
505 King Avenue
Columbus, OH 43201-2693

Vendor Approval

of

Test/QA Plan

For

Systematic Investigation of Fumigant Technologies for
Decontamination of Biological Agents from Contaminated Building Materials

VENDOR REPRESENTATIVE (SIGNATURE) DATE

VENDOR REPRESENTATIVE (PRINTED NAME)

COMPANY NAME

Battelle
505 King Avenue
Columbus, OH 43201-2693

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ANOVA	analysis of variance
atm	standard atmospheric pressure
B_0	laboratory blank
$B_{(t>0)}$	procedural blank
BEST	Battelle Eastern Science and Technology Center
BBRC	Battelle Biomedical Research Center
BSA	bovine serum albumin
BSC	biological safety cabinet
BSL	biological safety level
C	Celsius
$C_{(0)}$	positive control at time 0
$C_{(t)}$	positive control at time t
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	colony-forming units
cm	centimeter
CT	concentration x contact time
d	depth
DTT	dithiotheitol
EDTA	ethylenediaminetetraacetic acid
EPA	United States Environmental Protection Agency
h	height
H_A	alternate hypothesis
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ho	null hypothesis
HP	hydrogen peroxide
hr	hour
ISO	International Organization for Standardization
l	length
L	liter
LVS	live vaccine strain
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolarity
mol	mole
MREF	Battelle's Medical Research and Evaluation Facility
$N_{(t)}$	test coupon
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology

nm	nanometer
PBS	phosphate-buffered saline
PE	performance evaluation
ppm	parts per million
QA	quality assurance
QC	quality control
QMP	Quality Management Plan
RH	relative humidity
rpm	revolutions per minute
SD	standard deviation
SOP	standard operating procedure
STS	sodium thiosulfate
t	time
TBD	to be determined
TOPO	Task Order Project Officer
TSA	technical systems audit
TSB	tryptic soy broth
TTEP	Technology Testing and Evaluation Program
μg	microgram
μL	microliter
μM	micromolarity
w	width
$ZnCl_2$	zinc chloride

A3 Distribution List

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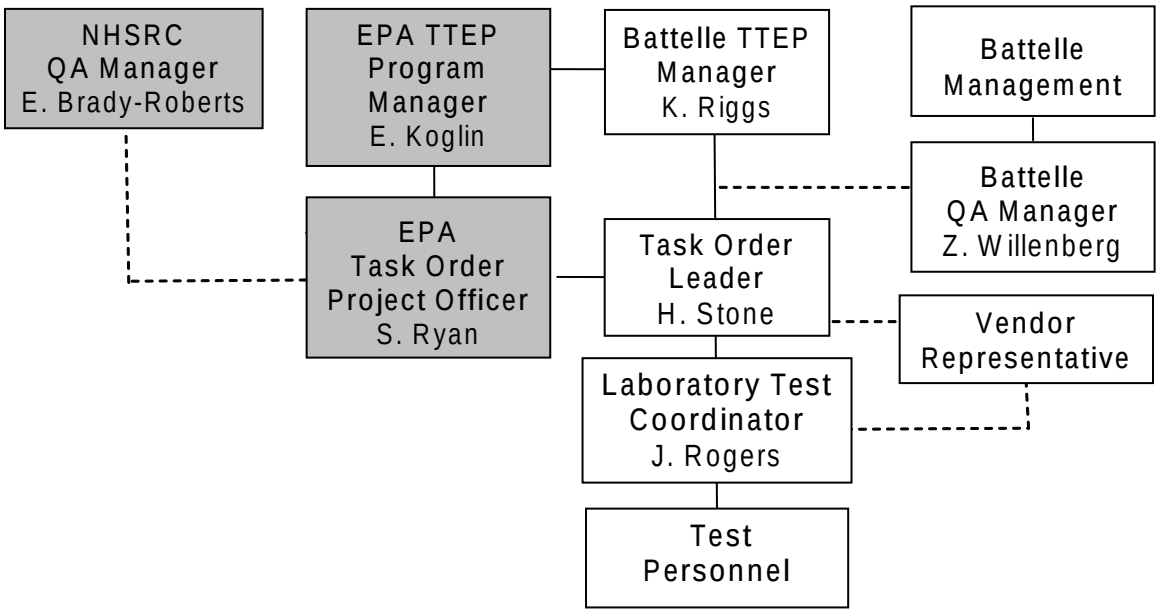
A4 Technology Investigation Organization

The technology investigation will be performed by Battelle under the direction of the U.S. Environmental Protection Agency (EPA) National Homeland Security Research Center (NHSRC) through the Technology Testing and Evaluation Program (TTEP). The organization chart (Figure 1) shows the individuals from Battelle, EPA, and vendors who will have responsibilities in the technology investigation. The responsibilities of these organizations and individuals are summarized in the following subsections.

Figure 1. Organization Chart for the Investigation of Fumigant Decontamination of Biological Agents



A4.1 Battelle

Dr. Harry Stone is Battelle's Task Order Leader for this technology investigation. He will have overall responsibility for ensuring that the technical, schedule, and cost goals established for testing and investigation are met and the procedures employed for testing are consistent with TTEP guidelines. Dr. Stone will serve as the primary interface for the Task Order Project Officer (TOPO). Dr. Stone's responsibilities are to:

- Ensure that TTEP procedures are being followed.
- Select the appropriate laboratory or location for the investigation.
- Prepare the draft test/quality assurance (QA) plan and draft report.
- Establish a budget and schedule for the technology investigation and direct the effort to ensure that budget and schedule are met.
- Revise this test/QA plan and draft report in response to reviewers' comments.
- Have overall responsibility for ensuring that this test/QA plan is followed.
- Keep the Battelle TTEP Manager informed of the progress and difficulties in planning and conducting the investigation.
- Prepare monthly technical and financial reports.
- Communicate with vendor representatives to secure vendor agreements.
- Respond to any issues raised in assessment reports and audits, including instituting corrective action as necessary.
- Coordinate distribution of final test/QA plan and final report.

Ms. Karen Riggs is Battelle's TTEP Manager. As such, Ms. Riggs will:

- Maintain communication with the EPA TTEP Program Manager on all aspects of the program.
- Monitor adherence to budgets and schedules in this work.
- Review and distribute monthly technical and financial progress reports.
- Review the draft test/QA plan and approve the final test/QA plan.
- Review the draft report.
- Ensure that necessary Battelle resources, including staff and facilities, are committed to the technology investigation.

Support Dr. Stone in responding to any issues that arise in assessment reports and audits.

- Issue a stop work order if audits indicate that data quality is being compromised.

Mr. Zachary Willenberg is Battelle's QA Manager for TTEP. As such, Mr. Willenberg will:

- Review the draft test/QA plan and approve the final test/QA plan.
- Conduct a technical systems audit (TSA) at least once during the technology investigation.
- Audit at least 10% of the investigation data.
- Prepare and distribute an assessment report for each audit.
- Verify implementation of any necessary corrective action.
- Notify Battelle's TTEP Manager to issue a stop work order if internal audits indicate that data quality is being compromised. Notify the Task Order Leader if such an order is issued.
- Provide a summary of the QA/quality control (QC) activities and results for the investigation report.
- Review the draft report.
- Ensure that all quality procedures specified in this test/QA plan and in the TTEP Quality Management Plan1 (QMP) are followed.

Dr. James Rogers is Battelle's Laboratory Test Coordinator for investigations conducted under this test/QA plan. His responsibilities are to:

- Assist in preparation of the draft test/QA plan.
- Arrange for use of the test facility.
- Arrange for the availability of qualified staff to conduct the investigation.
- Coordinate with vendor representatives to facilitate the performance of the investigation.
- Ensure that the investigation is conducted in accordance with this test/QA plan.
- Provide input into revision of this test/QA plan and report in response to reviewers' comments.
- Update the Task Order Leader on progress and difficulties in planning and conducting the investigation.
- Coordinate with the Battelle QA Manager for the performance of technical and data quality audits as required by Battelle or EPA Quality Management staff.

Test Personnel will conduct the work under this test/QA plan. Responsibilities are to:

- Conduct the systematic investigation in accordance with this test/QA plan and Battelle's policies and procedures.
- Accurately record all testing results and other information required by this test/QA plan and Battelle's policies and procedures.
- Provide input to the preparation of reports under this task order.
- Supports Battelle's Laboratory Test Coordinator.
- Conduct performance evaluation (PE) audits to assess the quality of measurements as required by Battelle.

A4.2 Vendors

Vendors will be invited to voluntarily submit their decontamination technologies for use in the testing under this test/QA plan. Vendors voluntarily submitting their decontamination technologies for use in the investigations will:

- Provide input for preparation of technology-specific amendments to the test/QA plan.
- Sign a vendor agreement specifying the respective responsibilities of the vendor and of Battelle in the investigation.
- Review and approve the final test/QA plan with any technology-specific amendments.
- Provide information on the quantitative response of their decontamination technology to aid in the planning of the investigation.
- Train Battelle and/or test facility staff in the operation of their decontamination technology, and sign documentation indicating this.
- If available, provide information regarding contact time and deposition requirements of the decontamination technology.
- Provide support, if needed, in use of their decontamination technology during testing.
- Review the respective draft report.

While vendor participation is preferred and confirmed by the vendor signing the test/QA plan, EPA may choose to test a technology even though the vendor declines to participate. For involuntary testing, EPA will secure the technology for use by Battelle in the investigation documented in this test/QA plan. At least one condition that is included in the investigation will be a condition published by or in documented communications from the technology vendor.

A4.3 EPA

Mr. Eric Koglin is the EPA TTEP Program Manager who directs Battelle's activities on the contract, "Testing and Evaluation of Homeland Security-Related Technologies for the Measurement, Sampling, Removal, and Decontamination of Chemical and Biological Agents" under which TTEP has been established.

Dr. Shawn Ryan is the EPA TOPO for Task Order 1123. As such, Dr. Ryan will:

- Have overall responsibility for directing the investigation process.
- Review the draft test/QA plan.

- Approve the final test/QA plan and any subsequent versions.
- Review the draft report.
- Select peer reviewers and coordinate EPA review process on the draft test/QA plan and report.
- Coordinate submission of report for final EPA approval.

Ms. Eletha Brady-Roberts is the NHSRC QA Manager for TTEP. As such, Ms. Brady-Roberts will:

- Review and approve the draft test/QA plan and any subsequent versions.
- Perform, at her option, one external TSA during each technology investigation.
- Notify the TOPO who will contact the Battelle TTEP Manager to issue a stop work order if an external audit indicates that data quality is being compromised.
- Prepare and distribute an assessment report summarizing the results of the external audit, if one is performed.
- Review the draft report.

A5 PROBLEM DEFINITION/BACKGROUND

Among its responsibilities related to homeland security, the EPA has the goal of identifying methods and equipment that can be used for decontaminating buildings following a terrorist attack using chemical or biological agents. In January 2003, EPA established the NHSRC to manage, coordinate, and support a wide variety of homeland security research and technical assistance efforts. In the interest of expanding our national readiness against highly ranked threat scenarios, the NHSRC, through TTEP, is conducting tests to investigate the performance of products, methods, and equipment for decontaminating porous and nonporous building materials contaminated with biological and chemical agents in order to assess how well the available decontamination tools will meet their performance objectives.

The amount of biological agent that remains on building materials following a terrorist attack could present a potential health risk for personnel reentering the building. The mission of TTEP Task Order 1123, enabled by this test/QA plan, is to gather data useful to all responders and decontamination decision makers on the efficacy and visual surface damage arising from use of various decontamination technologies.

The problem addressed by this test/QA plan is to answer the questions: How persistent are various biological agents (without undergoing decontamination) on various types of building materials to which they might be applied by terrorists? Regarding decontamination, what is the relationship between the type of biological agent, the type of material to which it is applied, the type of decontamination technology, and contact time for a given concentration (i.e., concentration x contact time; CT) on the log reduction in viability or bioactivity of biological agents? Is any visible damage observed on the building material surfaces arising from use of the decontamination technology?

The systematic investigation will generate objective persistence curves and efficacy curves, measured as log reduction in biological agent at various CT exposures, for various representative biological organisms or toxin applied to various porous and nonporous building materials.

This test/QA plan is applicable for testing and investigation of decontamination of biological agents using fumigant technologies under a variety of operating and environmental conditions. The scope described here was selected based on Battelle's initial recommendations for testing scenarios, agents, and scale of testing2, the needs of EPA, and discussions with the TOPO. This test/QA plan is specifically focused on decontamination of building materials typical of those found in public buildings or subways with the ultimate goal of providing technology for restoring the facility to a usable state.

A6 TECHNOLOGY INVESTIGATION DESCRIPTION AND SCHEDULE

The overall objective of the systematic investigation of decontamination technologies called for under this test/QA plan is to determine:

- Persistence of biological agents on indoor building materials.
- Efficacy of fumigant decontamination technologies for inactivating biological agents from representative porous and nonporous building materials under specific environmental conditions at multiple decontamination contact times.
- Visual surface damage arising from fumigant use.

For the testing conducted under this test/QA plan, quantitative efficacy assessment is accomplished by using sampling and analysis methods to determine the level of bioactive toxin or viable biological agent remaining on various building materials after treatment with fumigant decontamination technologies while monitoring and controlling parameters that may affect performance of the technology. Measurement of residual biological agent extracted from positive control coupons incubated under the same temperature and relative humidity (RH) conditions and for the same time period as the test coupons will provide the decontamination technology control data.

The investigation will:

- Generate data for biological agents that pose a threat to national security to show the persistence over time on building materials at specified environmental conditions.
- Generate data to show how efficacy of a specific fumigant against a specific type of biological agent is impacted by the type of material to which it is applied and the CT of the fumigant.
- Provide a qualitative evaluation of visible damage to the building material surfaces arising from use of the decontamination technology.

The fumigant decontamination technologies will be investigated for efficacy under specified environmental conditions, at five non-zero contact time periods, and against five biological agents. However, the number of biological agents, parameters of testing, and decontamination technologies may be changed by mutual agreement with Battelle and the TOPO. Key measurements in this test/QA plan include:

- Level of bioactive toxin (botulinum toxin type A) or number of viable organisms (*Yersinia pestis* CO-92, *Francisella tularensis* live vaccine strain [LVS], *Bacillus anthracis* Ames spores, and *Brucella suis* biotype I).
- Environmental conditions, including temperature and RH.
- Operating conditions, including fumigant CTs.

Qualitative observation of the condition of the test coupons before and after decontamination treatment and the overall ease of preparation, application, handling, and storage of the decontamination technology will be documented and included in the final report.

Systematic technology investigations will apply analytical techniques in novel situations. To ensure the quality of the findings, a variety of quality assessment tools will be applied. These tools include controls, replicates, equipment calibrations, TSAs, performance evaluation assessments, data quality audits, and external peer review of the test/QA plan and the reports. These tools are described in detail in appropriate sections of this test/QA plan.

All testing and investigation conducted through TTEP is under the direction of EPA and is subject to the TTEP QMP.[1] In investigating each technology, Battelle will follow the general procedures described in the TTEP QMP and this test/QA plan, and, as necessary, amend the test/QA plan for the specific type of decontamination technology being tested. The amendments will include a description of the specific fumigant decontamination technology being investigated and other modifications of the test/QA plan for the investigation of the specific technology. Modification may include, but is not limited to, biological agents, building materials, temperature, RH, and CTs.

The investigation described in this test/QA plan is expected to commence within four weeks after this test/QA plan has been approved and preliminary methods demonstrations have been completed. It is anticipated that a minimum of five weeks in the laboratory will be required to complete all systematic testing for botulinum toxin, five weeks for *Y. pestis*, five weeks for *F. tularensis*, five weeks for *B. anthracis*, and five weeks for *B. suis*. One week is needed between select agent or toxin to transition the testing to the next organism to be tested. The draft report will be submitted to the TOPO approximately seven weeks after completion of all laboratory testing.

Assuming that the test/QA plan is approved by June 1, 2007, the following schedule is anticipated:

- Kickoff June 8, 2007
- Method Development Completion, July 6, 2007
- Botulinum toxin Completion, August 17, 2007
- *Y. pestis* Completion, September 28, 2007
- *F. tularensis* Completion, November 9, 2007
- *B. anthracis* Completion, December 21, 2007
- *B. suis* Completion, February 15, 2008
- Draft Report Completion, April 4, 2008

A7 QUALITY OBJECTIVES AND CRITERIA

The objective of this test/QA plan is to establish laboratory test procedures to systematically investigate:

- Persistence on building materials of high-priority select agents and toxins that pose a threat to national security: botulinum toxin A, *Y. pestis*, *F. tularensis*, and *B. suis*. (*B. anthracis* Ames spores are not included in the persistence testing because persistence of the spores is well documented.)
- Efficacy, determined quantitatively, of using fumigant technologies to decontaminate those high-priority select agents and toxins (botulinum toxin A, *Y. pestis*, *F. tularensis*, *B. suis*, and *B. anthracis*) from representative porous and nonporous building material surfaces.
- Obvious damage to the building material surfaces arising from use of the fumigation technology.

The data quality criteria, shown in Table 1, provide criteria for determining the adequacy of data generated under this task for achieving the objectives: investigating persistence, efficacy, and damage.

Valid data will be assumed if measurements meet the performance criteria in Table 1 and the calibration and performance evaluation audits show acceptable results, as described in their respective sections of this test/QA plan. Accuracy is ensured by the calibration of the instruments, including micropipettes, microplate reader, thermometer, hydrometer, and clock, described in Section B7. The calibration frequency is shown in Table 9. In all cases the calibration is sufficient to meet or exceed the critical data quality objectives. The precision and/or accuracy performance evaluation audit of the instruments, counting of colony-forming units (CFUs), titration of ClO_2, and hydrogen peroxide (HP) measurements using the Hach HP Test Kit Model HYP-1 are described in Section C1.2 and will demonstrate that the laboratory performance data conform to the measurement performance criteria in Table 1. A performance evaluation audit will be performed once immediately prior to beginning testing.

Counts of positive controls and use of reference standards for instrument calibration will detect and allow correction of bias.

The representativeness of samples and comparability of data will be acceptable if test documentation confirms that the environmental conditions for test and control coupons are within specified limits (Table 1), coupons used as controls or test coupons are randomly selected and free of gross visual abnormalities on the test surface, and, for test and positive control coupons, identical procedures outlined in this test/QA plan are followed (within the limitations of the method) for application, environmental conditions, extraction, and measurement of biological agent in the extracts. Coupons will be visually examined and any with abnormalities on the test surface will be rejected from use as control or test coupons.

The completeness of the testing will be acceptable if the number of valid measurements for test and control coupons are as specified in Section B1.1 of this test/QA plan. The objectives of this systematic investigation conducted in support of the project mission will be met if:

- Persistence curves are developed from valid data that are within the constraints of the data quality objectives and QC requirements of this test/QA plan.
- Valid efficacy data are developed of the quality and quantity specified in this test/QA plan, i.e., data are within the constraints of the data quality objectives and QC requirements of this test/QA plan for each sampling moment specified in this test/QA plan.

Table 1. Data Quality Criteria for Measurement in the Decontamination Investigation

Data Required	Method	Unit	Measurement Performance Criteria	Corrective Action
Spike Volume	Micropipette	µL	Accuracy: ±5%	Replace with calibrated and sufficiently accurate micropipette; document the variance
Botulinum Toxin: Bioactivity	Microplate reader	Fluorescence at 423 nm	Precision: ±10% (controls) Accuracy: meets calibration standard (Table 9)	Replace with calibrated and sufficiently accurate microplate reader and reread plates
Colony-forming Units: *Y. pestis* *F. tularensis* *B. anthracis* *B. suis*	Manual count	CFU	Precision: ±10% (controls)	Provide training; test performance; recount questionable plates
pH	pH meter	pH units	Accuracy: 0.1 pH units	Replace pH standard solution; if readings of the pH standards remain inaccurate, replace pH meter with an accurate meter
Gas Volume	Calibrated mass flow controller Sierra Instruments) Stopwatch	Standard L/min min and second	Accuracy: ±5% Accuracy: ±1 second/ min	Replace the mass flow controller or stopwatch with a calibrated and accurate device; note variance in a laboratory data form
Temperature	Thermometer	°C	Accuracy: ±2 °C	Replace with calibrated and sufficiently accurate thermometer; document the variance
RH	Hygrometer	% RH	Accuracy: ±5% of full scale	Replace with calibrated and sufficiently accurate instrument; document the variance
Contact Time	Clock	hr	Accuracy: ±0.05% (2 second/hr)	Replace with calibrated and sufficiently accurate clock; document the variance
Chlorine Dioxide Concentration	Titration	ppm	Accuracy: ±10%	Determine whether the standard or titrant concentration is incorrect; replace standard or titrant; document the variance
HP Concentration	Hach HP Test Kit Model HYP-1	mg/L	Accuracy: ±10%	Replace test kit

The surfaces of the building materials will be visually (qualitatively) examined for damage after treatment with the fumigant; any damage will be documented as specified in this test/QA plan.

Bioactivity of biological agent (units of bioactivity of botulinum toxin or CFU of viable organisms) is the dependent variable in this investigation. Independent variables include the biological agents, building materials, temperature, RH, contact times, and concentration. In the measurement of the bioactivity, error is limited by the measurement performance criteria for the micropipettes with which specified volumes of suspensions of biological agent are applied, by accuracy requirements for counts of CFUs, and by accuracy and precision measurement criteria for the units of bioactivity of the toxin.

One of the critical steps in the determination of efficacy is the application of specific quantities of biological agent onto each test coupon ("spiking the coupon"). To know the amount applied, there must be known quantities of biological agents in a given volume of suspension, determined by application control measurements. As shown in Section B5, the bioactivity of botulinum toxin or number of *Y. pestis*, *F. tularensis*, *B. anthracis*, or *B. suis* CFUs spiked onto coupons will be acceptable if the measurement of application controls is within ±25% of the target spike level specified in Section B2.2. This ensures that the amount of biological agent being spiked onto coupons is consistent across tests.

Another critical step in the determination of efficacy is determining the residual units of bioactive botulinum toxin or CFUs of *Y. pestis*, *F. tularensis*, *B. anthracis*, or *B. suis* recovered in extracts from finished aluminum, computer keyboard keys, industrial carpet, and in painted wallboard paper. The amount of biological agent that can be recovered may be dependent on the specific material to which it is applied and the type of biological agent. Battelle has historical data only for B. anthracis on industrial carpet. Initial method demonstration, described in Section B1.1.2, will be performed to determine percent recoveries of the other combinations of biological agents and building materials new to Battelle (Table 2). Recovery at a given time *(i)* for a given material *(j)* is calculated as:

Equation 1. $$\text{Recovery}_j = \overline{C}_{(ij)}$$

Where:

$\overline{C}_{(ij)}$ is the mean number of viable organisms (CFU) or units of bioactivity of toxin recovered at the ith contact time from the jth material

The amount of biological agent applied to the building material coupon (CFU or bioactivity) equals the CFU or bioactivity/mL measured in the application control multiplied by the volume (mL) applied to the coupon. The calculation of % recovery is:

Equation 2. $$\%\ \text{Recovery}_j = \frac{\overline{C}_{(ij)}}{\text{Biological Agent Applied}}$$

Ten percent is the minimum acceptable percent recovery of biological agent from a given type of coupon extracted immediately prior to application of a fumigation technology. If the percent recovery is <10%, the TOPO may choose to modify acceptance criteria for percent recovery, request development of methods to increase the percent recovery, or discontinue use of this biological agent – material combination.

The data quality criteria, shown in Table 1, limit the error in CT by specifying measurement performance criteria for fumigant concentration and contact time. Confounds from temperature and RH are controlled by measuring the temperature and RH in the test and control chambers and adjusting the temperature and RH as necessary to maintain conditions within those specified in this test/QA plan. The temperature and RH will be monitored using calibrated methods meeting tolerances specified in Section B7 and passing the performance evaluation audit summarized in Section C1.2. As shown in Section C1.2, instruments used to measure these variables will have expected tolerances equal to or more stringent than those required to meet the data quality objectives shown in Table 1. Measurement criteria for QC samples are shown in Section B5.

Table 2. Test Matrix for Percent Recovery of Biological Agent from Building Materials

Biological Agent	Finished Aluminum	Keyboard Keys	Industrial Carpet	Primed, Latex Painted Wallboard Paper
Botulinum toxin	TBD	TBD	TBD	TBD
Y. pestis	TBD	TBD	TBD	TBD
F. tularensis	TBD	TBD	TBD	TBD
B. anthracis (Ames)	TBD	TBD	11% – 60%	50% – 51%
B. suis	TBD	TBD	TBD	TBD

TBD = to be determined

A8 SPECIAL TRAINING/CERTIFICATION

A8.1 General Site Description

Investigation of fumigant decontamination technologies will be conducted at the Battelle Biomedical Research Center (BBRC) [formerly Battelle's Medical Research and Evaluation Facility (MREF)] located in West Jefferson, Ohio, and the Battelle Eastern Science and Technology (BEST) Center located in Aberdeen, Maryland. These are both Battelle laboratories reporting to the manager of the BBRC.

Testing that requires use of *B. anthracis* Ames spores will be performed in a containment area designed to meet or exceed the BSL-3 facility guidelines published by the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health and entitled *Biosafety in Microbiological and Biomedical Laboratories* (5th edition, http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm). The BBRC and BEST Center investigate the performance of technologies for decontamination of pathogens and biotoxins. These facilities maintain state-of-the-art equipment, and professional and technical staffing expertise to safely conduct testing and investigation of hazardous biological materials. The BBRC and BEST Center operate in compliance with all applicable federal, state, and local laws and regulations and are routinely inspected by personnel from the appropriate government agencies. The BBRC and BEST Center are licensed to ship, receive, and handle select agents, as defined by CDC.

These facilities will meet all the requirements for safety, security, and testing capability established by this test/QA plan. Test procedures at these facilities are governed by established standard operating procedures (SOPs) that are specified by facility, number, and title. The investigation will be performed in accordance with Battelle's facility-specific methods and SOPs that are cited where appropriate throughout this test/QA plan.

A8.2 Training

Battelle staff will contact the vendor to obtain training or receive guidance on the use of the decontamination technology prior to the start of testing. Battelle will document this training or guidance. Biological agents included in this test/QA plan require special personnel requirements to perform some of the laboratory work. These special personnel requirements may include vaccinations, security clearances, and special training required for work with biological agents in the BSL-3 containment facility.

Because of the hazardous biological agents involved in this technology investigation, documentation of proper training is mandatory before testing takes place. Access to restricted areas of the test facility will be limited to staff who have met all the necessary training and security requirements. The requirements include Department of Justice and CDC select-agent program clearance as well as Occupational Safety and Health Administration requirements. Battelle's Laboratory Test Coordinator is responsible for ensuring that only properly trained and qualified personnel perform the work described in this test/QA plan.

All participants in this investigation (i.e., Battelle and EPA staff) will adhere to the security, health, and safety requirements of the Battelle facility in which testing will be performed. Vendor staff may train Battelle test personnel in the use of their technology but will not be the technology users during the testing. To the extent allowed by the test facility, vendor staff may observe, but may not conduct, any of the technology investigation activities identified in this test/QA plan.

The existing access restrictions of the test facility will be followed. No departure from standard procedures will be needed for this investigation. All visiting staff at the test facility will be given a site-specific safety briefing prior to the start of any test activities. This briefing will include a description of emergency operating procedures, and the identification, location, and operation of safety equipment (e.g., fire alarms, fire extinguishers, eyewashes, exits). Investigation procedures must follow all safety practices of the test facility at all times. Any report of unsafe practices in this investigation, by those involved in the investigation or by other observers, shall be grounds for stopping the investigation until the Battelle QA Manager and testing personnel are satisfied that unsafe practices have been corrected.

A9 DOCUMENTATION AND RECORDS

Documentation of training related to use of the decontamination technology, technology testing, laboratory analysis, field testing, data analysis, and reporting is maintained for all Battelle technical staff in training files at their respective locations. Vendor-specific training will be maintained with the study file. The Battelle QA Manager will verify the presence of appropriate training records prior to the start of testing.

The records for this investigation will include the test/QA plan, data collection forms, electronic files (both raw data and spread sheets), photographs, the draft and final reports and QA assessment reports. All of these records will be maintained by the Task Order Leader or his designee during the investigation and transferred to permanent storage at the conclusion of the investigation except for QA records, which will be maintained by the Battelle QA Manager.

B
Measurement and Data Acquisition

B1 EXPERIMENTAL DESIGN

This test/QA plan specifies procedures for investigating:

- Persistence — the percent recovery of biological agent over time from various types of indoor building materials under controlled environmental conditions.
- Decontamination efficacy — the log reduction in biological agent extracted from building materials following an experimental fumigation treatment compared to mean log reduction of biological agent extracted from coupons in the absence of the treatment (control).
- Fumigant damage — visual damage to the surface of building materials caused by the fumigation treatment.

Treatments for a given biological agent and building material will be defined in terms of the concentration of the fumigant (chlorine dioxide or HP), temperature, RH, and contact time. Differential efficacy of biological agents from various building materials may also be determined.

B1.1 General Test Design

The persistence testing will use a single group time series experimental design, diagrammed as:

R O_0 O_1 O_2 O_3 O_4 O_5

Where time passes from left to right and:

R = Random selection of the test coupons for each time point and type of biological organism.

O_t = Mean measurement (observation) of biological agent extracted from replicate coupons at time (t) = 0 and five subsequent time periods, designated by subscripts 1–5.

At a given point in time (t), the effect of time on persistence is O_t-O_0. The experimental design will allow the following null (H_O) and alternate (H_A) hypotheses to be statistically tested:

$$H_O: O_{(t)} - O_0 = 0$$
$$HA: O_{(t)} - O_0 < 0$$

That is, the experimental design will enable testing of the null or alternate hypothesis that, given an equivalent application of biological agent, the amount of biological agent on the coupons is constant, or, alternatively, decreases over time.

To determine the efficacy of the fumigation treatment on a biological agent, a pretest-posttest control group design will be used for each material, biological contaminant, and set of conditions, diagrammed as:

R O_1 X O_2
R O_1 O_3

Where time passes from left to right and:

R = Random selection of the test coupons for control, experiment, and type of biological organism.

O = Mean log reduction in measured biological agent extracted from replicate coupons

[$O_{1\ (Pretest)}$, $O_{2\ (Treatment)}$, and $O_{3\ (Control)}$].

X = Experimental variable, in this case the decontamination process.

At a given point in time, the effect of the experimental variable is $(O_2 - O_1)$-$(O_3 - O_1)$, or simplified, the effect of the experimental variable is $O_{2\ (Treatment)} - O_{3\ (Control)}$.

The experimental design will allow the following null (H_O) and alternate (H_A) hypotheses to be statistically tested:

$$H_O: O_{2\ (Treatment)} - O_{3\ (Control)} = 0$$
$$H_A: O_{2\ (Treatment)} - O_{3\ (Control)} > 0$$

For any particular material, the planned comparisons will include decontamination efficacy under given fumigant CT, and given environmental conditions (temperature and RH), for a particular biological agent. The experimental design will enable testing of the null hypothesis that there is no difference, or the alternate hypothesis that there is an increase, in the decontamination efficacy using the treatment compared to the control. The design will also enable comparison of rates of removal of biological agents from different material types under specific CT.

Method demonstration prior to performing the decontamination technology investigation will confirm that the proposed approach can be used effectively. Specifically, method demonstration will be used to determine percent recoveries of the biological agents from the material/biological agent combinations new to Battelle and the commercial method for quantifying botulinum toxin that, likewise, has not previously been used at Battelle. The method demonstration and acceptance criteria are described in Section B1.1.2.

The test methods will be refined based on the lessons learned during method demonstration and will be incorporated into standard test protocols that will be used to execute the test matrix. The results of the method demonstration will be discussed with the TOPO and recommended changes to the approach in this test/QA plan will be verbally approved by the TOPO prior to initiating Trial 1. (A “trial” is a single experimental condition, including, when practical, an identical absolute starting time.) An adaptive management approach will be used to incorporate new knowledge into the testing. Based on new knowledge, certain trials may not be performed or certain trials may be conducted at alternate conditions by agreement between Battelle and the TOPO.

The overall investigation process is shown in Figure 2. Persistence curves out to seven days will be developed for botulinum toxin A, and viable *Y. pestis*, *F. tularensis*, and *B. suis* on each of four building materials at controlled, ambient conditions (22 °C ± 2 °C and 35% – 45% RH). If temperature and RH specified for the second fumigant technology to be investigated under this test/QA plan differs substantially from the temperature and RH used for the first fumigation technology, at the discretion of the TOPO, persistence curves for biological agents (except *B. anthracis* spores) may be determined for such environmental conditions. Persistence curves will also be developed for the positive control coupons (maintained at temperature and RH conditions specified for the decontamination technology) for the five fumigation contact time points included in the respective decontamination tests.

After the persistence is analyzed, a decontamination curve for a given decontamination technology (e.g., chlorine dioxide and vaporized HP) will be determined for each of the five biological agents in combination with each of the four building materials. The fumigation technology will be applied against bioactive botulinum toxin type A, and viable *Y. pestis* CO-92, *F. tularensis* LVS, *B. anthracis* Ames, and *B. suis* biotype I. The building materials will be finished aluminum, computer keyboard keys, industrial carpet, and painted wallboard paper. However, the number of biological agents, parameters of testing, and decontamination technologies may be changed by mutual agreement between Battelle and the TOPO.

The experimental treatments (trials) expected to be performed under this test/QA plan are shown in Tables 3, 4, 5, and 6. For persistence and decontamination testing, positive controls are spiked with biological agent, but not exposed to the test conditions, and are analyzed at time zero. For decontamination testing, positive controls are also exposed to the same conditions (temperature, RH) and analyzed at the same time points as test coupons but without exposure to the fumigant. Figure 3 summarizes the treatment of test and control coupons; Section B5 provides details on the treatment of test and control coupons. During the persistence and decontamination testing, one laboratory blank and five positive control coupons will be extracted immediately prior to application of the fumigant technology to provide baseline percent recovery data; one procedural blank and five test coupons will be extracted at each non-zero time point. In addition, five positive control coupons will be extracted at each non-zero time point during the decontamination testing.

B1.1.1 Calculation of Recovery, Persistence, and Efficacy

Recovery of biological agents on various test materials will be determined after five time periods, as discussed in Section B1.1. Recovery [mean ± standard deviation (SD)] will be calculated for a given type of test material spiked with a given biological agent by dividing the number of viable organisms or bioactivity of toxin extracted from replicate test coupons at a given time by the number of coupons.

Statistical analysis will consist of evaluating whether the mean recovery of the biological agent at a particular contact time on a particular test material is statistically significantly different ($p < 0.05$) from the recovery of biological agent at time zero. Both point estimates and corresponding p-values will be produced for each comparison to test the null hypothesis.

Figure 2. Flow Diagram for Testing for Each Fumigant Under Task Order 1123

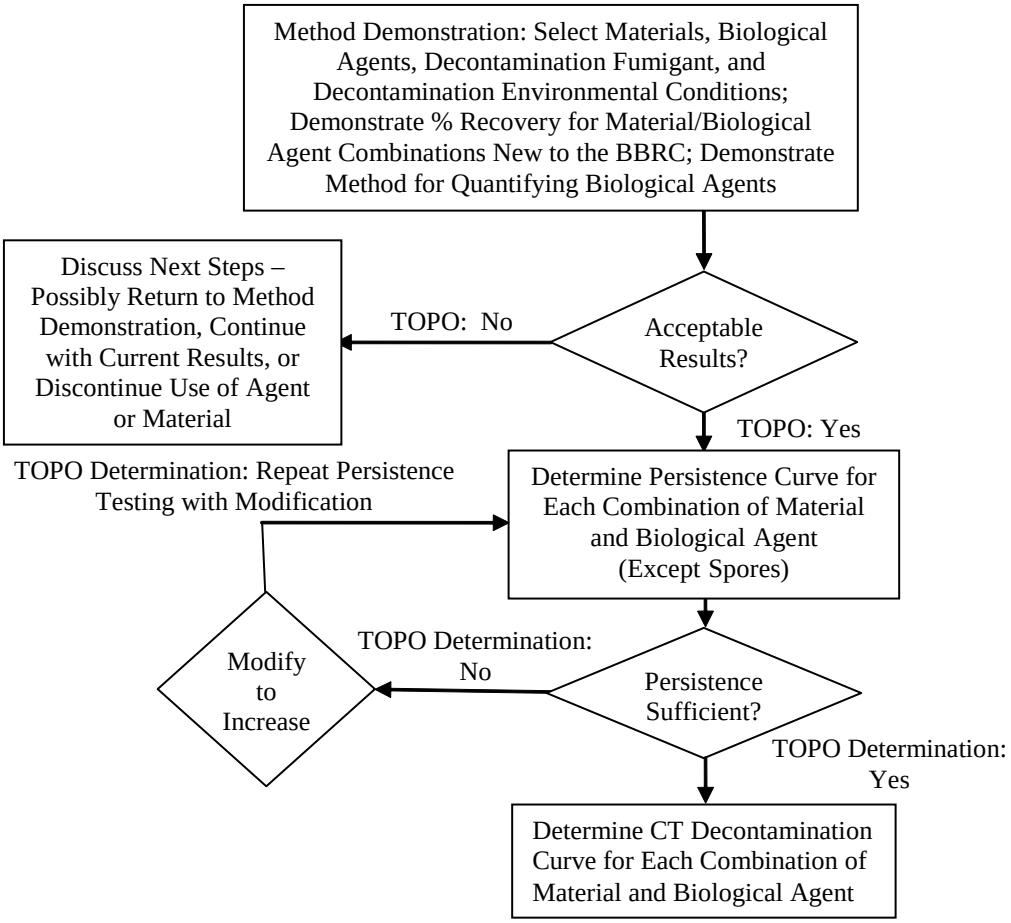
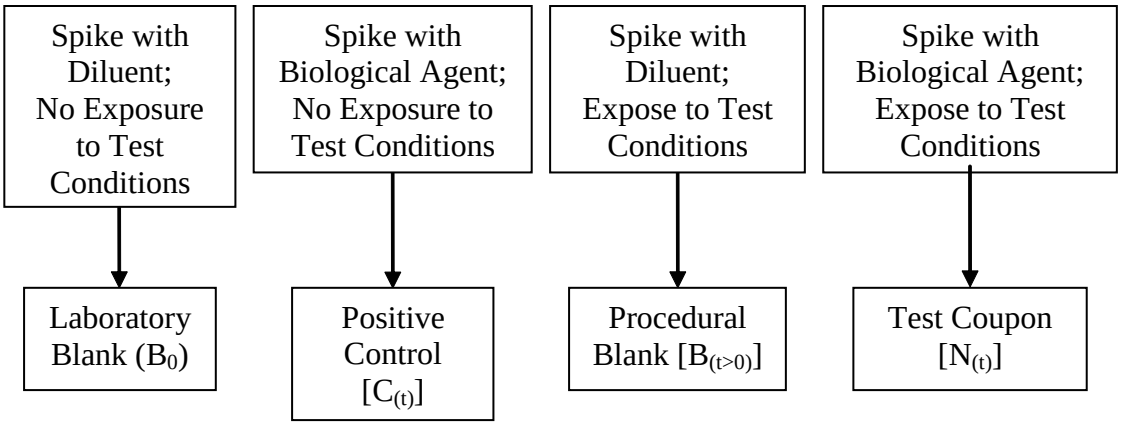


Figure 3. Summary of Treatments for Laboratory Blank, Positive Control, Procedural Blank, and Test Coupons



The primary persistence results from the coupon testing will be a persistence curve developed by graphing, on a semi-log scale, the recovery against time for a given biological agent on a given type of building material. Each point will show the mean bioactivity of toxin or number of viable organisms recovered along with a 95% confidence interval for each type of biological agent and building material.

The reduction in biological agent exposed to fumigation and the corresponding decontamination efficacy of the fumigant applied to various biological agents on various test materials will be determined after five time periods, as discussed in Section B1.1. The recovery results from the coupon testing will be an efficacy plot developed by graphing, on a semi-log scale, the amount of biological agent recovered against CT (contact time x the concentration of fumigant), for specific biological agent or toxin on a specific type of building material. Each point will show the efficacy along with a 95% confidence interval.

The first step in the calculation of overall efficacy is a separate calculation of efficacy for each individual coupon in a given set of replicates. Efficacy is defined as the extent (by log reduction) to which the agent extracted from the coupons after the treatment with the decontamination technology was less than what was extracted from positive control coupons (not exposed to the fumigant) maintained at the same temperature, RH, and time after being spiked with the same amount and type of biological agent as the treatment. Efficacy will be calculated for each test coupon within each combination of contact time (i) and test material (j) as:

Equation 3. $$Efficacy_{\mathbf{j}} = \log_{10}\left(\overline{C}_{\mathbf{j}}\right) - \log_{10}\left(N_{ijk}\right)$$

Where:

$\overline{C}_{ij}$ = arithmetic mean of the bioactivity of toxin or number of viable organisms recovered from control coupons at the *i*th contact time and *j*th test material.

N_{ijk} = bioactivity of toxin or number of viable organisms recovered on the *k*th replicate test coupon at the *i*th contact time and *j*th test material.

For living bioagents, cases may exist in which a very small number of viable organisms are observed on the replicate coupons after a given treatment. If these cases are observed, the data may be modeled using methods consistent with rare events such as Poisson distributions.

Statistical analysis will consist of evaluating whether the efficacy at a particular contact time on a particular test material is statistically significantly different (p. 0.05) from zero. Both point estimates and corresponding p-values will be produced for each comparison to test the null hypothesis.

The primary efficacy results from the coupon testing will be a plot developed by graphing the efficacy (log reduction) against contact time at a given concentration of fumigant for a specific biological agent on a specific type of building material. Each point will show the mean log reduction in CFU or bioactivity along with a 95% confidence interval.

The question of whether the fumigant causes surface damage is answered by a qualitative assessment of the differences in color, reflectivity, and roughness of the procedural blanks compared to the laboratory blank. (See Section B5 for a description of the treatment of procedural and laboratory blank coupons.) The surface damage will be accessed as a by-product of the investigation of the efficacy of decontamination technologies. An in-depth investigation of the effect of the decontamination treatment on the materials is beyond the scope of this test/QA plan. The presence or absence of obvious surface damage will be noted on a laboratory data form. If surface damage is observed, a second person will review the observations to confirm that there is obvious damage to the coupons. Representative photographs of the condition of the coupons before and after decontamination will be used to document findings. Photographs will be taken to document all types of observed damage. The photographs will be maintained in the permanent records from this investigation. The

primary results will be a table showing observed damage or, if no damage is observed, a statement to that effect in the report. The report will include pictures of representative coupons that were damaged in the decontamination process and corresponding coupons that were not exposed to the decontamination technology.

B1.1.2 Method Demonstration

A brief (three-week) method demonstration will be performed to ensure that the methods previously used are adequate for the novel biological agent-material combinations in this test/QA plan. The method demonstration will include determination of repeatability, reproducibility, and minimal method enhancements that may be considered to improve the percent recovery and variance. The method demonstration will also evaluate potential use of a commercially available fluorometric method (SNAPtide™) for measurement of the bioactivity of botulinum toxin.

Methods for Demonstrating Percent Recovery for Material/Biological Agent Combinations

Methods will be demonstrated for extracting and quantifying each biological agent from each type of indoor building material coupon that will be used in the testing and where Battelle has no prior experience with the material/biological agent combination. This will be done by spiking coupons with biological agent, allowing the agent to dry for a specified period of time (at specified temperature and RH conditions), extracting the coupons, and analyzing for the amount of biological agent recovered. The percent recovery analysis will be repeated for each type of biological agent and coupon combination where prior experience is lacking. These data will be used to ensure that at least 10% recoveries are achieved. This will be completed prior to beginning the persistence testing in Section B1.1.3.

1. Quantify the biological agent in the stock solution as described in Section B3.
2. Spike five coupons of one material with biological agent stock solution as described in Section B2.
3. Extract and quantify the biological agent from coupons as described in Sections B2 and B4.
4. The method will be acceptable if the stock solution is within ±25% of the target spike level of the biological agent specified in Section B1.4 and percent recoveries are >10% and <120% of CFU or bioactivity applied to the coupon.

Method for Demonstrating Method for Quantifying Botulinum Toxin

1. Calbiochem recommendations[3] for using the fluorogenic SNAPtide™ botulinum A substrate to measure the bioactivity of botulinum toxin will be followed.
2. A standard curve of the fluorescence measurement at various dilutions of the stock botulinum toxin will be graphed against the corresponding nanomoles of SNAPtide™ substrate cleaved by the toxin to determine units of bioactivity. One unit of bioactivity is defined as the amount of botulinum toxin needed to catalyze the release of 1.0 micromole of cleaved SNAPtide™ fluorophore from intact SNAPtide™ substrate per minute (min).

The preliminary results of method demonstration will be presented to the TOPO within two weeks after the data is generated.

B1.1.3 Experiments to Determine Environmental Persistence Curves

The matrix for the experiments to be performed for persistence testing is shown in Table 3. Persistence of botulinum toxin, *Y. pestis*, *F. tularensis*, and *B. suis* in the absence of a decontamination technology will be determined on each of the four building materials selected at the temperature and RH that will be used in the testing of the first fumigant. The temperature will be maintained at ±2 °C and RH will be maintained at ±5% of the target conditions. (Because the persistence of B. anthracis spores is well documented, its persistence is not investigated here.) Test coupons are spiked as described in Section B2.2. One laboratory blank and five test coupons will be extracted at time zero to provide baseline percent recovery data. At each selected time points, shown in Table 3, one procedural blank and five test coupons will be removed from the test chamber. The coupons will be extracted immediately as describe in Section B4.1.

If persistence is not sufficient to enable useful testing of the fumigant technologies, an approach for development of alternative methods for increasing persistence will be sought in the open and classified literature; recommendations will be provided to the TOPO (at his request). Alternative methods may then be adopted by mutual agreement between the TOPO and Battelle, and through amendment of this test/QA plan.

Table 3. Persistance Matrix at 22 °C ± 2 °C and 35% – 45% RH

Trial	Agent	Material	Period of Persistence					
			Time 0	2 hr	4 hr	8 hr	3 days	7 days
1	Botulinum toxin	Finished aluminum, computer keyboard keys, industrial carpet, and painted wallboard paper	O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1
2	*Y. pestis*		O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1
3	*F. tularensis*		O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1
4	*B. suis*		O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1

B_0 – not spiked with biological agent (laboratory blank); extracted at time zero
$B_{(t>0)}$ – not spiked with biological agent (procedural
$O_{(t)}$ – spiked with biological agent (test coupon)

B1.1.4 Conduct Experiments to Determine Persistence Curves at Alternate Environmental Condition

If environmental conditions, specified by the TOPO, for a fumigant technology to be investigated under this test/QA plan differ substantially from those in Trials 1–4 shown in Table 3, at the discretion of the TOPO, persistence curves for biological agents (except *B. anthracis* spores) may be determined for such environmental conditions prior to conducting the decontamination testing. The persistence curves will be determined for biological agents and building material combinations used in Trials 1–4 at the temperature and RH selected for the additional fumigation technology. The matrix for this persistence testing is shown in Table 4. The methods, materials, and organisms will be consistent with those used in Section B1.1.3.

At the TOPO's discretion, testing of alternative methods to alter persistence (optional subtask below) may be performed in lieu of testing described in this subsection.

Table 4. Persistance Investigation Matrix at Alternative Environmental Conditions

Trial	Agent	Material	Period of Persistence					
			Time 0	2 hr	4 hr	8 hr	3 days	7 days
5	Botulinum toxin	Finished aluminum, computer keyboard keys, industrial carpet, and painted wallboard paper	O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1
6	*Y. pestis*		O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1
7	*F. tularensis*		O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1
8	*B. suis*		O_0=5 B_0=1	O_1=5 B_1=1	O_2=5 B_2=1	O_3=5 B_3=1	O_4=5 B_4=1	O_5=5 B_5=1

B_0 – not spiked with biological agent (laboratory blank); extracted at time zero
$B_{(t>0)}$ – not spiked with biological agent (procedural blank); in chamber with test coupons
$O_{(t)}$ – spiked with biological agent (test coupon)

B1.1.5 Optional: Method Demonstration to Increase the Persistence of Biological Agent

If persistence of any biological agent is not sufficient to enable useful testing of the selected fumigant technology, the TOPO, in consultation with Battelle, may request development of alternative methods for preparing or inoculating the coupons to increase persistence. A plan for development of alternative methods will be provided to the TOPO for approval prior to initiating methods development under this optional subtask.

B1.1.6 CT Decontamination Curves for the Chlorine Dioxide Gas Generation Technology

The matrix for this testing, shown in Table 5, will develop data necessary to establish a chlorine dioxide decontamination curve for each biological agent, with the addition of *B. anthracis* Ames spores, and building material combinations investigated in Trials 1–4. The methods used to control, monitor, and record the chlorine dioxide concentration and the environmental conditions in the test chamber will be consistent with those previously used by Battelle[4, 5] The fumigant decontamination testing will use one fumigant concentration and five non-zero time points for each biological agent on each building material coupon combination. The fumigant decontamination will be performed at the concentration, temperature, and RH specified by the TOPO. Temperature and RH will be controlled as described in Section B1.5. Positive control and test coupons will be spiked in a single batch as described in Section B2.2. One laboratory blank (B_0) and five test coupons (N_0) will be extracted at time zero to provide baseline percent recovery data. The fumigant will be applied to the procedural blank and test coupons at the CTs selected in consultation with the TOPO. At each contact time, one procedural blank and five test coupons will be removed from the test chamber and five positive control coupons not exposed to the fumigant will be immediately extracted as described in Section B4.1.

Table 5. Decontamination Investigation Matrix for the Chlorine Dioxide Gas Generation Technology

Trial	Agent	Material	Fumigation Parameters: Concentration, Temperature, and %RH	Fumigant Contact Time, hr					
				Time 0	Time 1	Time 2	Time 3	Time 4	Time 5
9	Botulinum toxin	Finished aluminum, computer keyboard keys, industrial carpet, and painted wallboard paper	TBD ppm, 23 °C ± 2 °C, 70% – 80%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
10	*Y. pestis*		TBD ppm, 23 °C ± 2 °C, 70% – 80%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
11	*F. tularensis*		TBD ppm, 23 °C ± 2 °C, 70% – 80%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
12	*B. anthracis*		3000 ppm, 23 °C ± 2 °C, 70% – 80%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
13	*B. suis*		TBD ppm, 23 °C ± 2 °C, 70% – 80%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1

B_0 – not spiked with biological agent, not exposed to fumigant
$B_{(t>0)}$ – not spiked with biological agent, exposed to fumigant
$N_{(0)}$ – spiked with biological agent, not exposed to fumigant
$N_{(t>0)}$ – spiked with biological agent, exposed to fumigant

B1.1.7 CT Decontamination Curves for the Vaporized Hydrogen Peroxide Technology

The matrix for testing to establish a fumigant decontamination curve for each biological agent and building material combination, shown in Table 6, will be generally consistent with the approach described in Section B1.1.6, except that the fumigant CTs will be selected to be appropriate for the vaporized hydrogen peroxide (HP) technology being investigated. The ability to control, monitor, and record the fumigant concentration in the test chamber will be demonstrated prior to performing the experiments described in Table 6. The fumigant decontamination testing will use one concentration and five non-zero time points for each biological agent on each building material coupon combination. The HP system will be run in an automated cycle controlled by the commercial unit using the manufacturer's recommended parameters except contact time. The test chamber RH, fumigant concentration, and duration of decontamination cycle phases will be controlled by the HP system, if automated, or controlled by test personnel within the manufacturer's specifications (except for contact time). If an HP system manufacturer does not specify cycle parameters and/or the HP system is not fully automated to control cycle parameters, the fumigant concentration, cycle phases and duration, temperature, RH, and contact times will be selected in consultation with the TOPO and methods will be developed and demonstrated for test personnel to monitor and control these parameters.

B1.2 Scale of Testing, Testing Apparatus

The impact of the critical parameters, presented in Section A7, on decontamination efficacy will be evaluated through bench-scale testing in the laboratory. The test chamber used to expose test coupons to the fumigant (decontamination test chamber) will be a Compact Glove Box Model 830-ABC (Plas Labs, Inc., Lansing, MI; Figure 4). The glove box has internal dimensions of 71 cm w x 59 cm d x 74 cm h and external dimensions of 110 cm w x 61 cm d x 79 cm h, with a total volume of 317 L. The glove box also has a top opening of 43 cm x 58 cm and an attached transfer chamber that is 30 cm long and has an inner diameter of 28 cm (total transfer chamber volume of 18.5 L). Glove ports are available for working in the glove box. The test chamber (but not the control chamber) will be painted with black latex to shield the interior from light. Two 93-mm computer fans in the glove box provide air flow in the test chamber to promote uniform exposure to the fumigant throughout the chamber.

Table 6. Decontamination Investigation Matrix for the Vaporized Hydrogen Peroxide Technology

Trial	Agent	Material	Fumigation Parameters: Concentration, Temperature, and %RH	Fumigant Contact Time, hr					
				Time 0	Time 1	Time 2	Time 3	Time 4	Time 5
14	Botulinum toxin	Finished aluminum, computer keyboard keys, industrial carpet, and painted wallboard paper	TBD ppm, TBD ± 2 °C, TBD RH ± 5%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
15	*Y. pestis*		TBD ppm, TBD ± 2 °C, TBD RH ± 5%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
16	*F. tularensis*		TBD ppm, TBD ± 2 °C, TBD RH ± 5%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
17	*B. anthracis*		TBD ppm, TBD ± 2 °C, TBD RH ± 5%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1
18	*B. suis*		TBD ppm, TBD ± 2 °C, TBD RH ± 5%	N_0=5 B_0=1	N_1=5 C_1=5 B_1=1	N_2=5 C_2=5 B_2=1	N_3=5 C_3=5 B_3=1	N_4=5 C_4=5 B_4=1	N_5=5 C_5=5 B_5=1

B_0 – not spiked with biological agent, not exposed to fumigant
$B_{(t>0)}$ – not spiked with biological agent, exposed to fumigant
$N_{(0)}$ – spiked with biological agent, not exposed to fumigant
$N_{(t>0)}$ – spiked with biological agent, exposed to fumigant

The chamber used for persistence testing, and for positive control and laboratory blank coupons during decontamination testing, is a fabricated acrylic chamber identically configured to the glove box used for the test chamber. The persistence/control chamber will not be exposed to fumigants. When used as a control chamber for the decontamination testing, blank (i.e., spiked with diluent only, no spores) and positive control (i.e., spiked with spores but not decontaminated) coupons will be prepared for each type of material and will be maintained at the same temperature and RH for the same "contact" time period as the test coupons. These coupons will be used along with data from the test (spiked and decontaminated) coupons to determine decontamination efficacy.

The temperature and RH of both the test and control chambers will be adjusted, maintained, and monitored as described in Section B1.5.

The experiments will use test coupons that are approximately 1.9 cm w x 7.5 cm l. As was done in previous decontamination studies[6], multiple coupons of each building material will be spiked with the biological agent, placed horizontally on a wire rack in the custom modified glove box, and exposed to the fumigation technology at a specified temperature and RH. This testing will provide a highly controlled, reproducible approach to developing CT decontamination curves for the fumigant decontamination technologies.

Figure 4. Glove Box for Decontamination Testing

B1.3 Test Materials

The building materials to be used are:

- Finished aluminum
- Computer keyboard keys
- Industrial carpet
- Painted wallboard paper

As shown in Table 7, each of the test coupons is cut to 1.9 cm x 7.5 cm size (except for each of the computer keyboard key coupons, which are 1.3 cm x 1.3 cm size) from the interior of a large piece of test material. Edges and damaged areas will be avoided in cutting test coupons. Additional types of coupons or reference tests may be added by the TOPO through amendment of this test/QA plan.

Coupons will be sterilized by gamma irradiation at ~40 kiloGray. Individual (or multiple) coupons will be sealed in 6-mil Uline poly tubing (Uline, Chicago, IL), and this packaging will preserve sterility until the coupons are ready for use. This is intended to minimize contamination by microorganisms other than those being evaluated.

Table 7. Building Materials

Material	Manufacturer / Supplier Name	Lot or Batch Number / Description	Approximate Coupon Size on Test Surface, w x l
Finished Aluminum	TBD	TBD	1.9 cm x 7.5 cm
Computer Keyboard Keys	DataCal. Gilbert, AZ 85233	Medium Grey IBM shell blanks Acrylonitrile, Butadiene, and Styrene (ABS) Plastic	1.3 cm x 1.3 cm
Industrial Carpet	Carpet Corporation of America, Rome, GA	Style M 7978, color #910	1.9 cm x 7.5 cm
Painted Wallboard Paper	United States Gypsum Company	Set-E-493; Roll-30; 5-16-03 Roller painted on one side using Martin Senour Paints. One primer (#71-1185) and two finish (flat, #70-1001) coats; wiped with 70% isopropanol	1.9 cm x 7.5 cm

B1.4 Biological Agents and Toxin

The select agents and toxin used in the testing are:

- Botulinum toxin Type A (Sigma Catalog Number B8776, or equivalent)
- *Yersinia pestis* CO-92 (Battelle M-YPO166)
- *Francisella tularensis* LVS (OSU FTL361)
- *Bacillus anthracis* Ames spores (USAMRIID M-BAA202)
- *Brucella suis* Biotype I (Battelle BRU163)

The information in parentheses after bacteria names above refers to the specific Battelle stock used in this testing. The identity of these stocks was verified using Idaho Technologies, Inc. R.A.P.I.D® PCR-based identification.

Botulinum toxin (100 μg) will be reconstituted in 1 mL of phosphate-buffered saline (PBS).

The methods for culturing the bacteria follow. The density of bacteria or spores in the stock solution will be determined as described in Section B4.1.

A stock solution of *Y. pestis* will be prepared fresh in advance of each day of coupons that are spiked. Coupons are spiked by transferring a loop of *Y. pestis* to a tube containing 8 mL of tryptic soy broth (TSB) (or other nutrient medium). The TSB will be incubated at 26 °C ±

2 °C for 24–48 hr to establish a stock solution with an approximate concentration of 1.0×10^8 to 5.0×10^8 CFU/mL. Under these growth conditions, the resulting suspension is expected to be in log-phase growth.

A stock solution of *F. tularensis* will be prepared fresh in advance of each day of coupons that are spiked. Coupons are spiked by transferring a loop of *F. tularensis* to a tube containing 8 mL of cystine heart broth with 2% hemoglobin or thioglycollate broth. The medium will be incubated at 35 °C ± 2 °C in 5% CO_2 for 66–72 hr to establish a stock solution with an approximate concentration of 1.0×10^8 to 5.0×10^8 CFU/mL. Under these growth conditions, the resulting suspension is expected to be in log-phase growth.

B. anthracis spores will be prepared according to established BBRC procedures.[7] A primary culture of *B. anthracis* Ames spores from Battelle stock will be grown overnight (16–18 hr at 37 °C) in nutrient broth (BD Diagnostic Systems, Sparks, MD, USA) on an orbital shaker set at 150–200 rpm and used as an inoculum for a scale-up culture that will be grown in nutrient broth for 6–8 hr with the same orbital shaker setting as the primary culture. Leighton-Doi Broth (BD Diagnostic Systems) inside a BioFlo fermentor (New Brunswick Scientific Co., Inc., Edison, NJ) will be inoculated with the scale-up culture and left to grow for approximately 24 hrs at 37 °C. Cultures exhibiting >80% refractile spores will be centrifuged (fixed angle rotor) at approximately 10,000 – 12,000 x g for 15–20 min at 2 °C–8 °C. The resultant pellet will be washed twice, resuspended in ice-cold, sterile water, heat-shocked (incubation at 60 °C for 45–60 min), centrifuged, and washed at least twice to remove cellular debris. The spore preparation will be purified by centrifuging through a gradient of ice-cold, sterile 58% Hypaque-76 (Nycomed Amersham, Princeton, NJ, USA) at 9,000 x *g* for two hr at 2 °C–8 °C. The resultant pellet will be washed and resuspended in ice-cold, sterile water and evaluated by phase-contrast microscopy. Preparations containing >95% refractile spores with <5% cellular debris will be enumerated, diluted with sterile water to approximately 1.0×10^9 CFU/mL and stored at 2 °C–8 °C. Details of the method are published in the *Journal of Applied Microbiology*.[6]

A stock solution of *B. suis* will be prepared fresh in advance of each day of coupons that are spiked. Coupons are spiked by transferring a loop of *B. suis* to a tube containing 8 mL of TSB (or other nutrient medium). The TSB will be incubated at 35 °C ± 2 °C in 5% CO_2 for 24–48 hr to establish a stock solution with an approximate concentration of 1.0×10^8 to 5.0×10^8 CFU/mL. Under these growth conditions, the resulting suspension is expected to be in log-phase growth.

B1.5 Monitoring and Controlling Temperature and Relative Humidity

The experimental temperature and RH in both the test chamber and the control chamber will be monitored and controlled at the level specified in a given trial. The temperature and RH of the respective chambers will be measured to determine that the conditions specified for decontamination testing are achieved. A continuous monitoring system using HOBO U12 data loggers will be used for temperature and RH during decontamination. The HOBO U12 will be modified to protect the device from degradation by the fumigant, as recommended by the TOPO. A NIST-certified thermometer/ hygrometer will be used every 20 min to verify the data from the continuous monitoring system.

Prior to use in testing, the methods for measuring temperature and RH will be evaluated in the presence of fumigants to determine whether the fumigants interfere with the accuracy of the measurements. If the fumigants interfere with the RH measurement, other methods for measuring RH will be evaluated and selected for use in this testing.

An ultrasonic fog generator, inside of a "fogging chamber" will be used to raise the humidity in the test and control chambers. To raise the humidity in the test and control chambers, air from the test or control chamber will be pulled into the fogging chamber through an inlet. In the fogging chamber, the air will be humidified to ~100% RH using an ultrasonic fog generator or other device. The high-humidity air will pass out of the fogging chamber through a water trap to remove any liquid water and be returned to the test or control chamber. Mixing fans in the test or control chambers will cause the humidity to rapidly equilibrate at a higher RH.

To achieve low RH, the test or control chambers will be flushed with zero air until the target RH is reached.

A heating pad under the test and control chambers will be used to warm the air to the desired temperature in the test and control chambers and the fogging chamber.

B1.6 Application of Fumigant, Monitoring of Test Parameters

The test coupons intended for decontamination will be transferred into the test chamber for experimental treatment after inoculation. Fumigant will be introduced and monitored as described below. If the fumigant concentration in the test chamber falls below 90% of the target concentration, a calculated amount of fumigant will be introduced into the test chamber to prevent the concentration from falling below the acceptable range.

Positive control coupons and laboratory blanks are maintained undisturbed in a separate test chamber and incubated under the same temperature and RH conditions as the corresponding test coupons. They are processed at the same time points as the test coupons.

To conduct a given decontamination trial, the same general approach will be followed.

Initiate Testing:

1. Achieve desired temperature and RH.
2. Load the decontamination test chamber with test coupons and procedural blanks.
3. Load positive control coupons and laboratory blanks into the control chamber at the same temperature and RH as the test (fumigation) chamber.
4. Ensure the temperature and RH reach the specified conditions in the decontamination test chamber and the control (non-decontamination) chamber.
5. Introduce an appropriate amount of fumigant into the decontamination test chamber to achieve the concentration specified for a given trial.

Monitoring and Sampling:

1. Continuously monitor temperature and RH in the test and control chambers. Using an independent hygrometer and thermometer that is not continuously exposed to the fumigant, measure and record temperature and RH in the test chamber every 20 min and as soon as practical after removal of coupons; adjust temperature and RH as necessary to maintain specified conditions.
2. Sample fumigant every 20 min and as soon as practical after removal of coupons to determine concentration by withdrawing an aliquot of the decontamination test chamber's atmosphere; see Section B4.2 for details on monitoring fumigants.
3. Add fumigant to the decontamination test chamber as needed to maintain the specified concentration of fumigant. Confirm that the correct concentration has been achieved by replicate measurement.

Coupon Removal from the Chamber and Coupon Extraction:

1. At each specified contact times, remove appropriate coupons from the test and control chambers through the transfer chamber, and immediately place into individual tubes with extraction fluid.
2. Extract coupons and measure biological agent in the extract as described in Section B4.1.
3. After all coupons have been removed from the test chamber, flush the chamber with laboratory air.

B2 SAMPLING METHODS

B2.1 Coupons

The test coupons will be visually inspected prior to inoculation with the biological agents. Coupons with anomalies on the test (application) surface will be rejected from use. To prevent contamination of test surfaces, sterile technique following Battelle policies and guidelines[8-10] will be exercised during all phases of handling the coupons.

On each investigation day, each coupon will be assigned a unique identifier code for traceability by the test personnel. The identifier code will be placed on the coupons, vials, and plates in indelible ink.

The physical effect of the decontamination technology on the test coupons will be evaluated during the efficacy investigation procedure. The qualitative approach will provide a gross visual investigation of whether the decontamination technology damages the various building materials. Before and after decontamination of the test coupons, the appearance of the decontaminated coupons will be visually inspected, and any obvious changes in the color, reflectivity, or apparent roughness of the coupon surfaces will be recorded. This comparison will be performed for each of the test materials before extraction or sampling of the decontaminated test coupons. Photographs will be taken to document all types of observed damage. The photographs will be kept in the project files.

B2.2 Application of Biological Agents onto Coupons

Application of biological agents onto the test and positive control coupons will be performed in a Class II or III biological safety cabinet (BSC) according to Battelle procedures.[8] Test and positive control coupons will be placed lying flat in the cabinet and contaminated at challenge levels of approximately 10 μg of botulinum toxin or approximately 1×10^7 CFU of viable organisms (*Y. pestis*, *F. tularensis*, *B. anthracis*, or *B. suis*) per coupon. A 100-μL aliquot of a stock suspensions of approximately 100 μg/mL of botulinum toxin (or approximately 1×10^8 CFU/mL of *Y. pestis*, *F. tularensis*, *B. anthracis* spores, or *B. suis*) will be dispensed using a multichannel micropipette applied as 10-μL droplets in two rows across the surface of the coupon.

After the coupons are spiked with *B. anthracis* spores, they will be placed into the control chamber and dried overnight in a BSC-3 cabinet at ambient conditions (approximately 22 °C and 40% RH). After drying overnight, the coupons spiked with *B. anthracis* spores, along with specified blank coupons will be transferred into the test or control chamber at the temperature and RH that will be used for fumigation. Fumigation will begin immediately after the test coupons and procedural blank coupons are placed in the test chamber.

After the coupons are spiked with botulinum toxin, the coupons will be allowed to dry for one hour at ambient conditions (approximately 22 °C and 40% RH). After drying for one hour, the spiked coupons and specified blank coupons will be placed inside of the test or control chamber at the temperature and RH that will be used for fumigation. Fumigation will begin immediately after the test coupons and procedural blank coupons are placed in the test chamber.

After the coupons are spiked with *Y. pestis*, *F. tularensis*, or *B. suis*, the spiked coupons and specified blank coupons will be placed inside of the test or control chamber at the temperature and RH that will be used for fumigation. Fumigation will begin immediately after the test coupons and procedural blank coupons are placed in the test chamber. The time from spiking the coupons until fumigation begins will be as short as technically feasible, minimizing drying time.

B2.3 Confirmation of Surface Application Concentration

The stock suspension of botulinum toxin, *Y. pestis*, *F. tularensis*, *B. anthracis*, and *B. suis* used to spike the coupons will be reenumerated on each day of use following the procedures described in Section B4.1. During enumeration of the respective colonies, any colonies with morphological characteristics inconsistent with the target organism will be documented.

B3 SAMPLE HANDLING AND CUSTODY

Testing, including preparing samples, decontamination, and analysis, will occur within a secure area. The test materials will be extracted and assayed as described in Section B4. No special handling will be required to protect the samples from degradation during this brief transfer. Each coupon will be assigned a unique identifier code by the test personnel for traceability. Sterile technique following Battelle guidance[8-10] will be exercised in all handling of the coupons. No chain-of-custody record will be needed.

B4 ANALYTICAL METHODS

B4.1 Extraction and Quantification of Biological Agents

Each type of bioagent, test, positive control, and blank coupons will be placed into individual sterile 50-mL conical vials to which 10.0 mL of sterile extraction buffer will be added. PBS will be the extraction buffer for all of the biological agents except *B. anthracis*. PBS with 0.1% Triton X-100 (Sigma) will be the extraction buffer for *B. anthracis* spores.[5, 6] The tubes will be agitated on an orbital shaker for 15 min at approximately 200 revolutions per minute (rpm) at room temperature. Refined test methods, based on the lessons learned during the method demonstration, may be used, depending on discussions with the TOPO. Any changes made to these test methods will be documented in an amendment, as specified in the TTEP QMP.

B4.1.1 Method for Quantifying Botulinum Toxin

The testing will evaluate decontamination efficacy by measuring the amount of residual bioactive botulinum toxin on test and control coupons using the fluorogenic SNAPtide™ botulinum A substrate. The SNAPtide™ substrate is cleaved by botulinum toxin, releasing a fluorophore with an emission spectrum that can be monitored at wavelength 423 nm. The Calbiochem recommendations3 will be followed. Specifically, the toxin will be pre-incubated in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, 300 μM zinc chloride ($ZnCl_2$), 1.25 mM dithiotheitol (DTT), and 1 mg/mL bovine serum albumin (BSA) for 30 min at 37 °C ± 2 °C to activate the toxin.

Assays will be done in a 50-μL reaction mixture to which SNAPtide™ substrate is added. The method for adding the SNAPtide™ substrate and the amount to be added to the reaction mixture will be determined in the method demonstration. After incubation at 37 °C ± 2 °C, for times TBD in the method demonstration, the reactions will be terminated by placing the reaction tube into an ice bath and adding 100 μL of ethylenediaminetetraacetic acid (EDTA) solution [20 mM HEPES, 100 mM EDTA, pH 8.0]. The fluorescence will be measured at wavelength 423 nm using a microplate reader. A standard curve of the fluorescence measurement at various concentrations of botulinum toxin will be graphed against the corresponding nanomoles of SNAPtide™ substrate cleaved by the toxin to determine units of bioactivity. One unit of bioactivity is defined as the amount of botulinum toxin needed to catalyze the release of 1.0 micromole of cleaved SNAPtide™ fluorophore from intact SNAPtide™ substrate per min under the test conditions described here.

B4.1.2 Method for Quantifying *Y. pestis*

The testing will evaluate decontamination efficacy by measuring the amount of residual CFU of *Y. pestis* on test and control coupons. The CFU of *Y. pestis* on the coupon will be determined using a dilution plating approach. Following extraction, the extract will be removed and a series of dilutions up to 10^{-7} will be prepared in sterile water. An aliquot (0.1 mL) of the undiluted extract and each serial dilution will be plated onto tryptic soy agar (Remel, Inc.). The cultures will be incubated for 48–72 hr at 26 °C ± 2 °C. Colonies will then be counted manually and CFU/mL determined. The number of CFU/mL will be determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data will be expressed as mean + SD of the numbers of CFUs in the total extract. Plates exhibiting no growth will be returned to incubation at 26 °C ± 2 °C. After an additional four days (seven total days of incubation), the plates will be checked again to detect any delayed growth. Colonies will be identified based on their growth characteristics on the medium. Small grey-white to pale yellow colonies (1 – 2 mm) are observed at 48–72 hrs. Early colonies have a shiny surface described as "hammered copper." Later colonies have an irregular "fried egg" appearance.[11] Any observed contamination by nontarget organisms will be documented.

B4.1.3 Method for Quantifying F. tularensis

The testing will evaluate decontamination efficacy by measuring the amount of residual CFU of *F. tularensis* on test and control coupons. The CFU of *F. tularensis* on the coupon will be determined using a dilution plating approach. Following extraction, the extract will be removed and a series of dilutions up to 10^{-7} will be prepared in sterile water. An aliquot (0.1 mL) of the undiluted extract and each serial dilution will be plated onto cystine heart agar enriched with rabbit blood and antibiotics (Remel, Inc.).[12] The cultures will be incubated for 68–72 hr at 37 °C ± 2 °C at 5% CO_2. Colonies will then be counted manually and CFU/mL determined. The number of CFU/mL will be determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data will be expressed as mean + SD of the numbers of CFUs in the total extract. Plates exhibiting no growth will be returned to incubation at 37 °C ± 2 °C. After an additional seven days (10 total days of incubation), the plates will be checked again to detect any delayed growth. Colonies will be identified based on their growth characteristics on the medium. On cystine supplemented commercial blood culture media, *F. tularensis* colonies after 48 hrs are small (1 – 2 mm in diameter), flat with a shiny surface, white to gray to bluish gray, opaque, with a smooth, entire edge.[13] Any observed contamination by nontarget organisms will be documented.

B4.1.4 Method for Quantifying *B. anthracis*

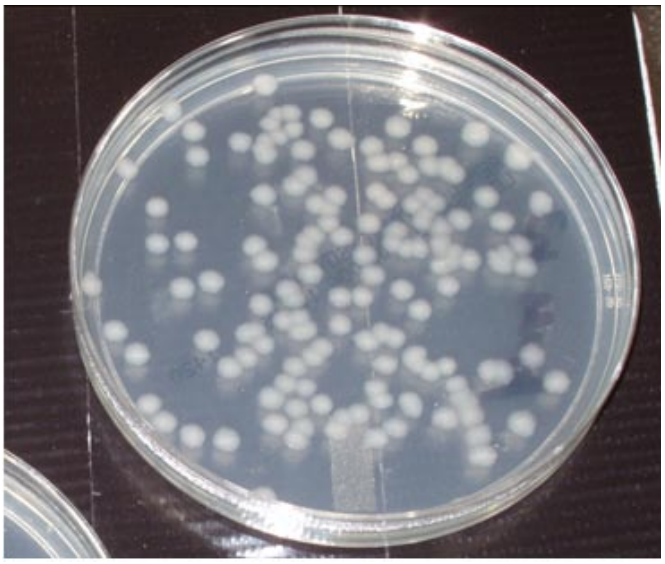
The testing will evaluate decontamination efficacy by measuring the amount of residual *B. anthracis* on test and control coupons.[5] Spores on the coupon will be determined using a dilution plating approach. Following extraction, the extract will be removed and a series of dilutions, at the TOPO's discretion, through 10^{-7} will be prepared in sterile water. At the TOPO's discretion, all samples will be plated onto tryptic soy agar monoculture plates. The following discussion assumes that tryptic soy agar will be used.

An aliquot (1.0 mL) of the undiluted extract and each serial dilution will be plated onto tryptic soy agar plates (Remel, Inc.) in triplicate. The cultures will be incubated for 18–24 hr at 37 °C ± 2 °C. Colonies will be counted manually and CFU/mL determined. The number of CFU/mL will be determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data will be expressed as mean + SD of the numbers of CFUs observed.

After incubation on tryptic soy agar for 18–24 hr at 37 °C, well-isolated colonies of *B. anthracis* are white, 2–5 mm in diameter. As shown in Figure 5, the flat or slightly convex colonies are slightly irregularly round with undulating edges and a ground glass appearance. Any observed contamination by nontarget organisms will be documented.

Potential confounding organisms will be excluded or controlled by sterilization of the coupons (described in Section B1.4) and use of sterile technique following Battelle guidance[8-10] blanks and a pure initial culture. Blanks will be run in parallel with the inoculated coupons (both test and positive controls). Assuming that the blanks show no CFUs and the morphology of the colonies is consistent with *B. anthracis* Ames, the CFUs observed from inoculated coupons will indicate surviving spores from the inoculated organisms.

Figure 5. *B. anthracis* Ames Colonies on Tryptic Soy Agar



B4.1.5 Method for Quantifying *B. suis*

The testing will evaluate decontamination efficacy by measuring the amount of residual CFU of *B. suis* on test and control coupons. The CFU of *B. suis* on the coupon will be determined using a dilution plating approach. Following extraction, the extract will be removed and a series of dilutions through 10^{-7} will be prepared in sterile water. An aliquot (0.1 mL) of the undiluted extract and each serial dilution will be plated onto tryptic soy agar (Remel, Inc.). The cultures will be incubated for 48 hr at 35 °C ± 2 °C in 5% CO_2. Colonies will then be counted manually and CFU/mL determined. The number of CFU/mL will be determined by multiplying the average number of colonies per plate by the reciprocal of the dilution. Data will be expressed as mean + SD of the numbers of CFUs observed. Plates exhibiting no growth will be returned to incubation at 35 °C ± 2 °C. After an additional five days (seven total days of incubation), the plates will be checked again to detect any delayed growth. Colonies will be identified based on their growth characteristics on the medium. After 48 hr, colonies of *B. suis* are small (1 – 2 mm in diameter) and round, honey-colored (when viewed through transparent medium), convex, and pearly white when viewed from above, translucent, with smooth margins.[15] Any observed contamination by nontarget organisms will be documented.

B4.2 Analytical Methods for Monitoring Fumigants

B4.2.1 Monitoring Chlorine Dioxide

Levels of chlorine dioxide in the test chamber will be monitored before beginning and during an experiment approximately every 20 min using a titration method. For consistency across TTEP task orders, the sampling method

will follow the procedures used in TO 1113[16] and TO 1114[17] and the colorimetric titration method will follow the standard operating procedures.[16] (The method is based on the Standard Method 4500-ClO2 E Amperometric Method II as recommended and used by Sabre Technical Services.) For this titration method, air from the test chamber is drawn through impingers (at a rate of 1 L/min using an air mass flow controller) that contain 15 mL of 5% potassium iodide in phosphate buffer (pH 7.0) solution. The pH of the impinger solution will be measured and recorded before use. Under these conditions chlorine dioxide oxidizes iodide to iodine and reduces chlorine dioxide gas to the chlorite ion, which dissolves in solution.

Chlorite ion does not react at neutral pH. After collection and reaction of the chlorine dioxide gas, the impinger solution will be acidified and the chlorite will be allowed to react further with the iodide ion, forming additional iodine and reducing the chlorite to chloride. The pH of the impinger solution will be measured and recorded immediately before titration. The total resulting iodine will be reduced to iodide when titrated against standard 0.1 normal [equal to 0.1 molar] sodium thiosulfate (STS). Molecular iodine appears yellow-brown in aqueous solution. The titration endpoint will be determined when the color of the solution changes from yellow-brown to colorless. The volume (mL) of STS solution titrated will be proportional to the amount of iodine generated, which is proportional to the gas-phase chlorine dioxide concentration in the air that passed through the impinger. Using the formula below, the concentration of chlorine dioxide may be calculated:

Equation 4. $$ClO_2\ (ppm) = \frac{V_1(mL) \times M}{V_2(L)} \times \frac{1}{5} \times 24.45 \times 1000$$

Where:

ClO_2 = chlorine dioxide (in ppm volume in air)

V_1 = volume of STS titrant (mL)

M = molarity (mol/L) of STS titrant (which for STS is equal to its normality)

V_2 = volume of air (at 25 °C, 1 atm) that passed through impinger (L)

24.45 = ideal gas constant, L/mol, at 25 °C, 1 atm

1000 = conversion factor = 106 ppm x 1 L / 1000 mL

Certified NIST- traceable chlorite standards, appropriately diluted in solution comparable to the sampling solution, will be titrated each day of chlorine dioxide sampling to verify accuracy. QC checks for the titration method are shown in Table 8.

B4.2.2 Monitoring Vaporized Hydrogen Peroxide

HP systems with automatic internal controls will monitor and regulate the concentration of HP in the test chamber at levels specified by the manufacturer.

In addition to any internal monitoring by the HP system, HP will be monitored using the method previously demonstrated by Battelle.[18] Every 20 min an impinger sampling moment will occur. The impinger will contain 15-mL carbon-filtered, double-deionized, organic-free water. For each sampling moment, sample flow will be maintained through the impinger at 1.0 L/min for four min. A Hach HP Test Kit Model HYP-1, Cat. No. 22917 (Loveland, CO) will be used to determine the HP concentration. Prior to analysis, diluted HP standards will be prepared and a five-point calibration curve will be generated to determine the number of drops of STS titrant required to decolorize the blue starch-iodine solution produced from a known concentration of aqueous HP. Three replicates will be made of each titration. For analysis, impinger solutions will be diluted to 500 mL with deionized water in Class A volumetric glassware. Each drop of STS titrant corresponds to ~81 ppm HP. The data from the HYP-1 analysis will verify the concentration reported by the generation system and enable monitoring of HP concentration variability from trial to trial.

B5 QUALITY CONTROL REQUIREMENTS

The representativeness and uniformity of the test materials are critical attributes to ensure reliable test results. Representativeness means that the materials used are typical of those used in buildings in terms of quality, surface characteristics, structural integrity, etc. Uniformity means that all test pieces are essentially equivalent for the purposes of testing. Representativeness will be ensured by selection of test materials that meet industry standards or specifications for indoor use and by obtaining those materials from appropriate suppliers. Uniformity will be maintained by obtaining a large enough quantity of material that multiple test samples can be obtained with presumably uniform characteristics (e.g., test coupons will all be cut from the interior rather than the edge of a large piece of material) or by using standardized coupons where available.

Quantitative standards do not exist for biological agents. Quantitative determinations of organisms in this investigation do not involve the use of analytical measurement devices. Rather, the CFU will be enumerated manually and recorded. Critical QC checks are shown in Table 8. The acceptance criteria were set at the most stringent level that can be routinely achieved and are consistent with the data quality objectives described in Section A7. Battelle will include application controls, positive controls, and blanks along with the test samples in the experiments so that well-controlled quantitative values can be obtained. Five replicate coupons will be included for each set of test conditions. Standard operating procedures using qualified, trained, and experienced personnel will be used to ensure data collection consistency. The confirmation procedure, controls, blanks, and method validation efforts will be the basis of support for biological investigation results.

Potential confounding organisms will be excluded or controlled by sterilization of the coupons described in Section B1.3 and use of sterile technique following Battelle guidance,[8-10] blank controls, and a pure initial culture. Sterile technique will be used to ensure that the culture remains pure. Blank controls will be run in parallel with the

inoculated coupons. Assuming that the blank controls show no CFUs, the CFUs observed from inoculated coupons will indicate surviving spores from the inoculated organisms.

If a high level of variability is observed, the test results will be discussed with the TOPO and a replicate test may be performed or corrective analytic actions may be taken.

B6 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The equipment needed for the investigation will be maintained and operated according to the quality requirements and documentation of the BBRC and BEST Center. Battelle has and follows a maintenance schedule for laboratory equipment. Equipment includes BSCs, incubators, and orbital shakers, which are calibrated annually by an outside contractor. Incubator temperatures are also recorded daily. During each day of testing, the equipment used is recorded including the date of calibration. Only properly functioning equipment will be used; any observed malfunctioning will be documented and appropriate maintenance or replacement of malfunctioning equipment will restore proper functioning.

B7 INSTRUMENT CALIBRATION AND FREQUENCY

The BBRC and BEST Center have standard operating procedures for the calibration of all laboratory equipment. A list of all equipment requiring calibration is maintained in a database, and calibrations are scheduled by designated staff. All equipment will be verified as being certified, calibrated, or validated at the time of use. Calibration of instruments will be done at the frequency shown in Table 9. Any deficiencies will be noted. The instrument will be adjusted to meet calibration tolerances and recalibrated within 24 hr. If tolerances are not met after recalibration, additional corrective action will be taken, possibly including the replacement of the equipment.

B8 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Supplies and consumables will be acquired from reputable sources and will be NIST-traceable when possible. Supplies and consumables will be examined for evidence of tampering or damage upon receipt and prior to use, as appropriate. Supplies and consumables showing evidence of tampering or damage will not be used. Coupon anomalies will be handled as described in Section B2.1. All examinations will be documented and supplies will be appropriately labeled. Project personnel will check supplies and consumables prior to use to verify that they meet specified task quality objectives and do not exceed expiration dates.

Table 8. Quality Control Checks

QC Sample	Information Provided	Acceptance Criteria	Corrective Action
Application Control	Calculated value of biological agents applied without confounds from building material or extraction impacting bioactivity.	±25% of the target application level. For spores and toxin; ±1 log for vegetative bacteria.	Reject results; prepare stock solutions meeting target application level in application control.
Laboratory Blank (coupon spiked with diluent without biological agent and not subjected to the test conditions)	Controls for sterility of coupon.	No observed CFU or units of bioactive botulinum toxin.	Reject results; identify and remove source of contamination.
Procedural Blank (coupon spiked with diluent without biological agent and subjected to the test conditions)	Controls for sterility of coupon during the test.	No observed CFU or units of bioactive botulinum toxin.	Reject results; identify and remove source of contamination.
Positive Control (coupon spiked with biological agent but not subjected to the test conditions)	Controls for percent recovery and confounds arising from history impacting bioactivity; controls for special causes.	>10% and <120% recovery of inoculated spores, organisms, or bioactivity of toxin; Grubbs outlier test (or equivalent).	<10% or >120% recovery: reject results. Outlier: evaluate/exclude value.
Test Coupon (inoculated and subjected to test conditions)	Controls for special causes.	Grubbs outlier test (or equivalent).	Outlier: evaluate/exclude value.
Blank Tryptic Soy agar/ Cystine Heart Agar Plate, Sterility Control (plate incubated, but not inoculated)	Controls for sterility of plates.	No observed growth following incubation.	Incubate additional 10 plates. If any additional growth is observed, reject results from the lot.
Blank Tryptic Soy Agar/ Cystine Heart Agar Plate, Growth Control (plate incubated after inoculation with organisms)	Controls for ability to support growth.	In the verification of application organism density, described in B2.3, ±25% of nominal organism density is observed.	Incubate additional 10 plates, including 5 from a different lot. If significant differences in growth are observed between the lots, reject results from the lot that is not adequately supporting growth.
Chlorine Dioxide Titration Method	Certified NIST-traceable chlorite standards, appropriately diluted in solution comparable to the sampling solution, titrated each day of chlorine dioxide sampling to verify accuracy.	±10% of standard value.	If accuracy does not meet acceptance criterion, determine and correct the cause of the variance.
Hach HYP-1 Kit	Calibrated each day of HP sampling to verify accuracy.	±10% of standard value.	If accuracy does not meet acceptance criterion, determine and correct the cause of the variance.

Table 9. Instrument Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
Hygrometer	Compare to independent hygrometer value once per quarter	RH ± 5% full scale
Thermometer	Compare to independent NIST thermometer (a thermometer that is recertified annually by either NIST or an International Organization for Standardization [ISO]-17025 facility) value once per quarter.	±1 °C
Micropipettes	All micropipettes will be certified as calibrated at time of use. Pipettes are recalibrated by gravimetric evaluation of pipette performance to manufacturer's specifications every six months by supplier (Rainin Instruments).	±5%
pH meter	Perform a 2-point calibration with standard buffers that bracket the target pH.	± 0.1 pH units
Calibrated mass flow controller (Sierra Instruments)	Compare against mini-Buck™ (NIST-traceable) primary flow calibrator prior to beginning fumigation of each type of biological agent being tested; repeat calibration annually.	±5%
Stopwatch	Compare against NIST official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java	±1second/min
Clock	Compare to official U.S. time @ www.NIST.time.gov every 30 days.	±1 min/30 days
Molecular Devices SPECTRAmax PLUS384 microplate reader	Performance verified monthly (when in use) by validation test plate, which is calibrated annually.	Pass each validation test performed when in use

B9 NONDIRECT MEASUREMENTS

There are no data needed for this evaluation that are obtained from nonmeasurement sources such as computer databases, programs, literature files, or historical databases.

B10 DATA MANAGEMENT

Data acquisition during the evaluation includes proper recording of the procedures used in the testing to ensure consistency in the evaluation and adherence to this test/QA plan; documentation of sampling/testing conditions; and recording of analytical results and evaluation conditions. Data acquisition (temperature, RH, and time) by Battelle testing staff will be carried out manually. Manual data acquisition will be recorded immediately in a consistent format throughout all investigations. All written records will be in ink and any corrections to recorded data will be made with a single line through the original entry. The correction will then be entered, initialed, and dated by the person making the correction. Any non-obvious correction will include a reason for the correction. Strict confidentiality of data will be maintained.

Relevant data for each test procedure and trial will be entered into an electronic spreadsheet set up to organize the data in a clear and consistent manner. The accuracy of entering manually recorded data into the spreadsheets will be checked at the time the data are entered, and a portion of the data will be checked by the Battelle QA Manager as part of the data quality audit (Section C1.3).

The testing results will be compiled in an investigation report. The report will briefly describe the TTEP and testing procedures, as well as all test data and observations. The preparation of the investigation report, review of the draft investigation report, revision of the draft investigation report, final approval, and distribution of the investigation report will be conducted as stated in the TTEP QMP.[1]

C
Assessment and Oversight

C1 ASSESSMENTS AND RESPONSE ACTIONS

C1.1 Technical Systems Audit

Battelle's QA Manager or designee will perform at least one TSA during the evaluation. The audit is to ensure the evaluation is performed in accordance with the TTEP QMP[1] and that the test/QA plan and that QA/QC procedures are implemented. The QA Manager, or designee, will review evaluation methods, compare test procedures to those specified in this test/QA plan, and review data acquisition and handling procedures. The QA Manager will prepare a TSA report, and the findings must be addressed either by modification of test procedures or by documentation in the investigation records and final report. At EPA's discretion, EPA QA staff may also conduct an independent on-site TSA during the evaluation. The EPA audit findings will be communicated to test personnel at the time of the audit and documented in a TSA report. These findings must be addressed as stated above.

C1.2 Performance Evaluation Audits

A PE audit will be conducted to assess the quality of the measurements made in this technology investigation. This audit addresses only those reference measurements that factor into the data used for analysis, including CFU count, temperature, RH, time, chlorine dioxide and HP concentrations, and optical density values from the microplate reader. The audit will be performed once during the technology evaluation and will be performed by analyzing a standard that is independent of standards used during the testing. Table 10 summarizes the PE audit that will be done and indicates the acceptance criteria for the PE audit.

No performance evaluation audit will be performed for biological agents and surrogates, as quantitative standards for these materials do not exist. The confirmation procedure, controls, blanks, and method validation efforts will be the basis of support for biological evaluation results.

In the event that results of analysis of the PE audit standard do not meet the acceptance criteria, the equipment will be recalibrated and then the PE audit standard will be reanalyzed. Continued failure to meet the PE audit criteria will result in the pertinent data being flagged and the purchase of new standards for recalibration of the equipment for repetition of the PE audit. Battelle's QA Manager will assess PE audit results.

C1.3 Data Quality Audit

Battelle's QA Manager will audit at least 10 percent of the evaluation data. The QA Manager will trace the data from initial acquisition, through reduction and statistical comparisons, and to final reporting. All data analysis calculations will be checked.

C1.4 Peer Review

External peer reviewers, coordinated by the TOPO, will review the test/QA plan and the report. In addition, the TOPO and NHSRC QA Manager will review the test/QA plan and the report. The TOPO will consolidate the comments and forward them to Battelle. Battelle will incorporate the comments into corresponding final test/QA plans and reports.

Table 10. Performance Parameters to Be Audited

Parameter	Audit Procedure	Expected Tolerance
CFUs	Compare to independent count of colonies	±10%
Temperature	Compare to independent thermometer value	±1 °C
pH meter	Measure a standard buffer not used to calibrate the pH meter	±0.1 pH units
Calibrated mass flow controller (Sierra Instruments)	Compare against mini-Buck™ (NIST-traceable) primary flow calibrator	±5%
Stopwatch	Compare against NIST official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java	±1second/min
RH	Compare to independent hygrometer value	±5%
Time (minutes)	Compare time to independent clock	±1 min/30d
Chlorine dioxide concentration	Titration of standard solution	±10%
HP concentration	Hach HYP-1 HP test kit	±10%
Optical density and wavelength	Compare optical density measurement of the microplate reader to standard	Optical density ± 1.0%, Wavelength ± 1 nm
Volume	All micropipettes will be certified as calibrated at time of use. Pipettes are recalibrated by gravimetric investigation of pipette performance to manufacturer's specifications every six months by supplier (Rainin Instruments).	±5%

C2 REPORTS TO MANAGEMENT

Each assessment and audit will be documented in accordance with the TTEP QMP[1], except that the TOPO will coordinate the peer review process and consolidate comments, which are forwarded to Battelle for incorporation into document revisions.

Assessment reports will include the following:

- Identification of any adverse findings or potential problems
- Space for response to adverse findings or potential problems
- Possible recommendations for resolving problems
- Citation of any noteworthy practices that may be of use to others
- Confirmation that solutions have been implemented and are effective

During the course of any assessment or audit, the QA Manager will identify to the technical staff performing experimental activities any immediate corrective action that should be taken. If serious quality problems exist, the QA Manager will contact the Battelle TTEP Manager, who is authorized to stop work. Once the assessment report has been prepared, the Building Decontamination Technology Area Leader or Task Order Leader will ensure that a response is provided for each adverse finding or potential problem and will implement any necessary follow-up corrective action. The QA Manager will ensure that follow-up corrective action has been taken. Copies of the assessment reports will be provided to EPA.

D
Data Validation and Usability

D1 DATA REVIEW, VALIDATION, AND VERIFICATION REQUIREMENTS

The data review, validation, and verification requirements include:

- Verification that all testing is completed as specified in the test/QA plan
- Ensuring that each data point is valid, i.e., complies with acceptance criteria specified in the test/QA plan
- Records generated during the investigation will receive a QC/technical review before these records are used to calculate, analyze, or report results.
- All data analysis calculations will be checked before the results are incorporated into the draft report.

D2 VALIDATION AND VERIFICATION METHODS

Section D of this test/QA plan provides a description of the validation safeguards

employed for this technology evaluation. Data validation and verification efforts include the performance of TSA and data quality audits as described in Section C.

The Task Order Leader and Laboratory Test Coordinator will compare the data generated to the requirements of the test/QA plan to ensure that all testing is completed in accordance with the plan.

The required review of records generated during the investigation will be performed by a Battelle technical staff member other than the person who originally generated the record. Test personnel will be consulted as needed to clarify any issues about the data records. The review will be documented by the person performing the review by adding his/her initials and date to a hard copy of the record being reviewed. This hard copy will then be returned to the Battelle staff member who generated or who will be storing the record. The data generated in this investigation will be transferred from laboratory notebooks and data collection forms into an electronic database. Data quality audits will be performed as specified in Section C1.3. Battelle's statisticians will then analyze the data as follows.

The efficacy data generated in this investigation will be fit to a two-factor fixed effects analysis of variance (ANOVA) model. There will be separate ANOVA models for each biological agent. Model diagnostics will be examined to assess whether there are any difficulties with outliers or the model assumptions of constant variance and normality of the residuals. If the data are not found to be adequate for the model, appropriate transformations or more general statistical models (e.g., nonparametric) will be considered.

The primary decontamination efficacy results from the coupon testing will be a matrix table in which each entry shows the mean log reduction in bioactivity of toxin or viable organisms along with a 95% confidence interval for each combination of biological agents, building materials, and contact times. A CT decontamination curve will be developed by graphing the mean efficacy (with a 95% confidence interval) against the CT for each type of biological agent and building material.

Statistical analysis as outlined in the experimental design in Section B1 will consist of determining whether the efficacy of the decontamination treatment at a particular contact time and test material was statistically significantly different from zero (null hypothesis). Additional comparisons may be made such as mean efficacy differences between materials. Both point estimates and corresponding p-values will be produced for each comparison.

In the analysis of persistence and efficacy, the Grubbs test[19] or equivalent test will be used to identify outliers. Outliers will be further investigated, but unless an error in recording or processing the data can be identified, the outlier will be excluded in the final analysis and noted in the report. The modeling and analysis will be carried out with PROC mixed in SAS v9.2 or later version.

All calculations and data included in models will be checked before the results are incorporated into the draft report.

D3 RECONCILIATION WITH DATA QUALITY OBJECTIVES

Data obtained during investigation of the decontamination technology as described herein, and statistically analyzed as described in Section D2, will be assessed by comparing with the Data Quality Objectives contained in Section A7. The Data Quality Objective regarding calculation of efficacy of the decontamination technology will be achieved and the data will be valid, providing the test data obtained for both positive controls and inoculated, decontaminated test coupons fall within the limits specified by the data quality indicators specified in the test/QA plan and therefore can be utilized to calculate decontamination efficacy. Invalid data will be rejected from use, and the test will be repeated to generate sufficient valid data to complete the test. The Data Quality Objective for observing the efficacy of the decontamination technology at various CT can be achieved, providing data compliant with the applicable data quality indicators specified in the test/QA plan are duly recorded during the investigation. The results of reconciling the data obtained with the data quality indicators will be discussed in the final report.

The preparation of the final report, the review of the report by vendors and others, the revision of the report, final approval, and distribution of the report will be conducted as stated in the program TTEP QMP[1], except that the TOPO coordinates

the peer review process and consolidates comments, which are forwarded to Battelle for incorporation into document revisions.

This test/QA plan generates data and analyses based on bench-scale tests under highly controlled conditions. Completion of the test/QA plan is expected to generate data that indicate the persistence of biological agents and the efficacy of fumigants. However, results should not be assumed to accurately reflect efficacy or persistence at larger scales, with other biological agents or preparations, at other environmental conditions, or when the biological agent is applied to other types of materials.

E
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