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This TOP supersedes TOP 08-2-065 Developmental Testing of Liquid and Gaseous/Vaporous Decontamination on Bacterial Spores and Other Biological Warfare Agents on Military-Relevant Surfaces, dated 11 February 2016.

Marginal notations are not used in this revision to identify changes, with respect to the previous issue, due to the extent of the changes.

14. ABSTRACT

This TOP describes standardized decontamination test practices and technical requirements to characterize microbes, quantitatively measure decontamination efficacy on materials contaminated with biological microbes, distinguish between static and cidal decontaminant activities, and to define practical data confidence by directly comparing controls at every step of the testing procedure.

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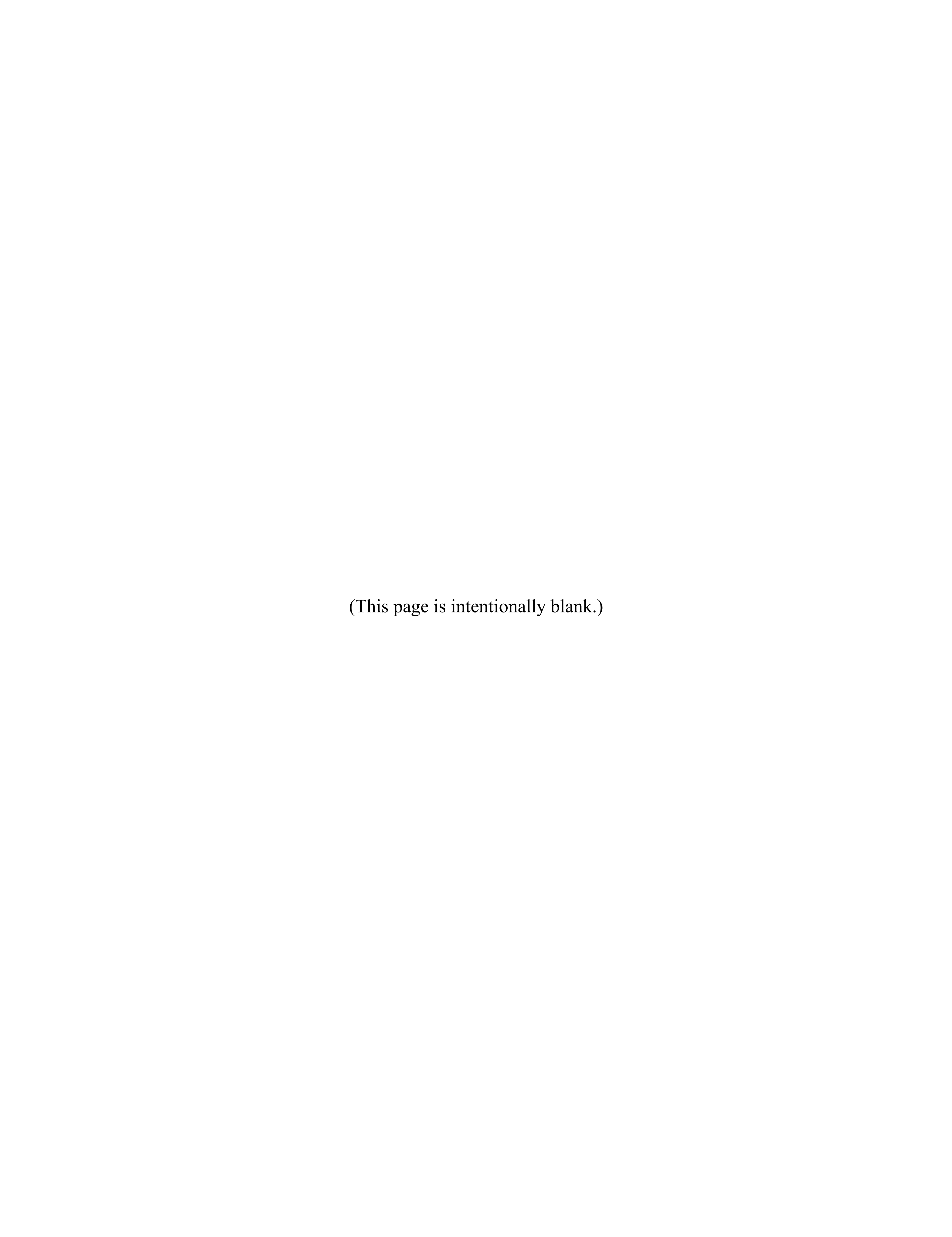

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US ARMY TEST AND EVALUATION COMMAND
TEST OPERATIONS PROCEDURE

Test Operations Procedure 08-2-065A 13 April 2021
DTIC AD No.

STANDARDIZED TEST PRACTICES TO QUANTITATIVELY MEASURE LIQUID, SOLID, AND VAPOROUS DECONTAMINANT EFFICACY ON MATERIALS CONTAMINATED WITH BACTERIAL SPORES OR OTHER BIOLOGICAL WARFARE AGENTS

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*This TOP supersedes TOP 08-2-065, dated 11 February 2016.
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1. SCOPE.

1.1 Purpose.

a. The purpose of this Test Operations Procedure (TOP) is to quantify the decontamination efficacy of vaporous, liquid, or liquids made from solid decontaminants after microbes (spores, vegetative cells or virus) are deposited onto coupons made from porous and non-porous materials. The procedures can distinguish between cidal and static activities. This is important because many decontaminants contain both reactive chemicals and high concentrations of bacteriostatic surfactants (this type of surfactant leaves the cell viable, but unable to replicate). All test samples are compared to pre-neutralized controls (or non-treated controls in the case of vaporous decontaminants), un-inoculated negative growth controls and solution controls on the same day as the test in order to increase practical confidence in the inactivation data.

b. This TOP provides guidance on test design and data requirements. The TOP guidance will be focused by information found in the test documentation including, but not limited to, the Operational Evaluation Plan, the Test and Evaluation Master Plan, the Capability Development Document (CDD), the CDD updates, and the Validated Online Lifecycle Threat.

c. This TOP provides guidance on procedures for microbe characterization, surrogate selection spore recovery and test standardization. This guidance is to address requests for standardization as described in the Government Accountability Office (GAO) Report to Congress 18-422 (September 2018)[**1] after the investigation of live irradiated spores that were shipped internationally from 2004-2015. Several manuscripts describing standardized practices were and are being published[2, 3, 4, 5]. A memorandum to the biological select agents and toxins (BSAT) Biosafety Program Office (BBPO), Department of Army, to address the GAO requests for standardization was submitted[6]. These reports include collaborative testing between U.S. Army Medical Research Institute for Infectious Diseases [(USAMRIID) and Edgewood Chemical and Biological Center] U.S. Navy (Dahlgren and Navy Medical), Joint Program-Guardian, Defense Threat Reduction Agency (DTRA), Food and Drug Administration (FDA) and over 150 representatives within American Society for Testing and Materials (ASTM) International. Hence, the test guidance here is applicable for developmental testing and is to be implemented to the greatest extent possible. If developmental testing is significantly different from standardized testing, then correlation testing to describe, characterize, and quantify all the differences must be conducted.

d. For the purposes of this TOP, the term biological agent refers to bacterial spores, vegetative bacteria, and viruses that are surrogates or select agents.

** Superscript numbers and letters correspond to those in Appendix E References.

1.2 Limitations.

a. This TOP is limited to currently approved standards, methods, and procedures. The procedures herein may not be sufficient to assess the efficacy of all decontaminants and decontamination processes. Development in practices, equipment, and analysis may necessitate new procedures. Additionally, test methods and standards must be adjusted as technology advances.

b. These procedures can be modified to assess the needs of the decontaminant and/or decontamination process as necessary. Any modification must be documented and recorded. As a result, test procedures and parameters listed in this TOP will require updating to accommodate any of the aforementioned changes that may occur.

c. The test procedures in this TOP are based on vaporous and liquid decontaminants. The procedures may need modification to accommodate other types of decontaminants (e.g., slurry).

d. The results obtained by using these test procedures are not designed to correlate with specific medical or toxicological values.

1.3 Procedure Development.

a. The following types of decontaminants were used to develop the procedures in this TOP:

(1) Vaporous decontaminants [e.g., hydrogen peroxide vapor; and hot, humid air (HHA)].

(2) Liquid decontaminants (e.g., electronically generated Chlorine Dioxide ($eClO_2$); Dahlgren Decon (First Line Technology, Chantilly, Virginia); and pH-adjusted sodium hypochlorite solution [pH of 5-8]).

b. The procedures in this TOP were developed using the following biological organisms: *Bacillus anthracis* (Ba) spores, *Bacillus thuringiensis* (Bt) spores, *Francisella philomiragia* (Fp) vegetative cells, MS2 bacteriophage, and Φ6 bacteriophage. If other biological agent species or strains are selected for a test, modifications to portions of these procedures may be necessary depending on the strain chosen. The strain(s) used will depend on availability and the discretion of the testing facility in coordination with the test sponsor, and must be documented.

2. FACILITIES AND INSTRUMENTATION.

2.1 Facilities.

Item	Requirement
Biosafety Level (BSL)-1, BSL-2 and/or BSL-3 laboratories	The laboratory provides the general support needed for work with biological agents, including analysis support, emergency response

	provisions, and hazardous waste storage and disposal.
It is strongly recommended that users consult the Biosafety in Microbiological and Biomedical Laboratories (BMBL)[7]	The biological laboratory must be constructed to ensure safe and secure storage, handling, general, and specialized biological analysis, and decontamination of biological agents and/or surrogates used for test and evaluation.
	The laboratory is required to store and prepare test quantities of biological agent surrogate materials, to charge disseminating devices, to prepare samplers, and to analyze all biological agent surrogates.

NOTE: The risk factors associated with the organism being tested will determine the BSL required. Depending on the organism and its strain, a BSAT registered facility may be required for testing.

2.2 Equipment.

Item	Requirement
Class II Biological Safety Cabinet (BSC) for biological organisms or surrogates	All BSCs must be certified and operating within the manufacturer's parameters. BSCs must be equipped with an air intake system and an exhaust system that exhausts through high-efficiency particulate air (HEPA) filters (i.e., capable of retaining 99.97 percent of particles 0.3 μm in diameter or larger). The BSC must be large enough to allow free air circulation around the test item. All applicable BSAT[8] regulations will be followed. BSCs or environmental chambers will be used to house the test item(s) during agent or surrogate contamination/decontamination and sampling. Airflow control is required.

2.3 Instrumentation.

Testing with vaporous or liquid decontaminants may require a contained (fully enclosed and under engineering controls) environment. Vaporous decontaminants will most likely require environmental control to ensure optimal efficacy. Liquid decontaminants may not require the same environmental restrictions. Environmental control during the test process may also be required to represent an operational scenario.

Parameter	Measuring Device	Permissible Error of Measurement
Air temperature	Thermocouple or other.	± 2.0 °Celsius (°C).
Relative Humidity (RH)	Humidity probe or other.	± 5 percent.
Airflow speed	Anemometer.	Within manufacturer's specification.

2.4 Test Controls.

Parameter	Tolerance
Air temperature	± 3.0 °C
RH	± 5 percent

3. REQUIRED TEST CONDITIONS.

3.1 Documentation.

a. A test plan will be developed using the test documentation and other program-specific requirements documents. The test plan will include a detailed statistical design of experiments, an execution matrix, and a data management plan. The test plan must be approved before testing can begin.

b. Relevant test site Standing Operating Procedures or other procedures will be reviewed for applicability, completeness, and adequacy.

c. All pertinent documentation will be maintained as part of the test record.

3.2 Biological Organism Strain Selection.

The determination of whether to use biological agents or surrogates will be made during the planning phase. Cost, schedule, and resources will dictate the amount of agent testing that can be performed. The acquisition, holding, preparations and/or testing of virulent strains require Center for Disease Control and Prevention (CDC) and/or Animal and Plant Health Inspection Service registration in the United States.

3.3 Biological Agent Preparation.

a. Spore and vegetative bacterial cell stock preparation.

(1) Bacterial stocks will be obtained from American Type Culture Collection (ATCC®). Viability and concentration will be monitored by performing plate counts for each organism. With each plate count, purity will be assessed by examining and evaluating colony

morphology for any stock contamination. Biological agent purity will also be evaluated (where applicable) by microscopy.

(2) Spore preparations should yield $\geq 1.0 \times 10^8$ colony-forming units (CFU) mL^{-1} in sporulation medium at the time of harvest, and able to concentrate to $\geq 1.0 \times 10^9$ CFU mL^{-1} in storage medium. Spore preparations should contain ≥95 percent phase-bright spores with less than 5 percent cellular debris as measured by phase-contrast microscopy. Enumeration will be performed using dilution plating, and should include a non-ionic surfactant such as 0.1 percent Tween 80 (Tween 20 and Triton™ X100 have also been used) during dilution. A comparison of heat-sensitive and heat-resistant (65 °C, 30 minutes) CFU concentrations will confirm that the spore preparation is ≥95 percent heat-resistant spores. Appropriate instrumentation, such as a Coulter counter (Beckman Coulter Incorporated, Indianapolis, Indiana) should be used to measure the size uniformity of spores. The challenge concentration must be documented in the test plan and report.

(3) Vegetative cell preparations should yield $\geq 1.0 \times 10^8$ CFU mL^{-1} in growth medium at the time of harvest, and able to concentrate to $\geq 1.0 \times 10^9$ CFU mL^{-1} in storage medium. The challenge concentration must be documented in the test plan and report.

b. Virus Stock Preparation. Virus preparations should yield $\geq 1.0 \times 10^8$ plaque-forming units (PFU) mL^{-1} in growth medium at the time of harvest, and able to concentrate to $\geq 1.0 \times 10^9$ PFU mL^{-1} in storage medium. Viability and concentration will be monitored by performing plaque assays for each viral stock. Virus purity is defined as having a single virus. Virus purity is not to be confused with any additives/components needed to stabilize the virus. The challenge concentration must be documented in the test plan and report.

c. The spore and vegetative cell preparations are for the production of high purity, lab-grade spores and vegetative cells. Clean spores and vegetative cells are used to determine baseline decontamination test data. However, any microbe can be mixed with debris to explore the performance envelope and increase confidence that a decontaminant will work in different environments. Since numerous debris classes and types exist and some debris can neutralize chemical decontaminants, tests should demonstrate that debris does not adversely affect the organism. Debris characterization and justification should be documented.

d. Debris or other additives that are deliberately added to microbes before inoculation should be described and documented. For example, spores were mixed with humic acid suspended in a spent sporulation medium before hot humid air decontamination in order to test the impact of organic debris on decontamination kinetics.

e. Test materials considerations:

(1) Coupon material is to be selected according to the claims or intended use of the decontaminant. Coupons may be made of any material - hard, flexible, porous, non-porous, metallic, or non-metallic. Flat (2-centimeter (cm) × 2-cm) coupons are preferred; however, non-flat coupons and smaller coupons can be tested. Confirm that the coupons can fit within 50-mL conical tubes. If filter units that fit within 50-mL conical tubes are used for testing, then coupons should be sufficiently thin to fit within the filter units[9,10].

(2) It is important to understand that the physical characteristics of the materials to be tested will have a significant impact on the recovery of biological agent from those materials. Some of the test material or biological agent characteristics that affect biological agent recovery include, but are not limited to, static charge, inherent stickiness, hydrophobicity, hydrophilicity, porosity, and permeability. An extraction efficiency study must be performed on each material and biological organism combination to be evaluated if no institutional data exists.

f. The minimum required challenge concentrations selected for this TOP are 1.0×10^7 CFU per sample area (e.g., 10 cm^2) for bacterial spores, 1.0×10^8 CFU per sample area (e.g., 10 cm^2) for vegetative cells, and 1.0×10^8 PFU per sample area (e.g., 10 cm^2) for viruses. If a different concentration is used (based on test document requirements or test sponsor requirements), the rationale for the change must be documented in the test plan and report.

3.4 Safety.

a. The procedures in this TOP should be performed only by those trained in microbiological techniques, and those who are familiar with antimicrobial agents and the application instructions of antimicrobial products. This TOP does not address all of the safety concerns, if any, associated with its use. It is the responsibility of the site to establish appropriate safety, health practices, and determine the applicability of regulatory limitations before use.

b. The determination of which biosafety level containment is required will be based on the biological organism strain(s) selected for evaluation. The most current edition of the BMBL[7] published by the CDC should be referenced regarding levels of containment.

c. It is the responsibility of the test site to follow all safety guidelines and to be knowledgeable about these procedures. Individual user(s) working with BSAT will undergo a security risk assessment and personnel suitability assessment, be immunized as appropriate, and be in compliance with Select Agent regulations.

3.5 Environmental.

a. It is the responsibility of the test site to establish appropriate environmental practices and determine the applicability of regulatory limitations before use.

b. Use test site procedures for handling the waste generated by testing to ensure environmental compliance.

3.6 Quality Assurance and Quality Control (QA/QC).

a. It is recommended that a QA/QC plan is developed for each test program, ensuring all appropriate records are maintained throughout the duration of testing and product evaluation. Items to consider including in a QA/QC plan, but are not limited to: viability and stability of biological agents and surrogates, purity and stability (pot life, shelf life) of decontaminants, calibration and maintenance of instrumentation and equipment, accuracy and precision of laboratory equipment, and quality and consistency of test articles.

b. The test equipment should be calibrated in accordance with the manufacturer's specifications for each piece of applicable equipment. All instrumentation should be covered by a valid certificate of calibration traceable to a national standard and operated in strict accordance with the manufacturer's handbook.

c. Test material(s) should be inspected as specified in the test plan, or in accordance with the procedures in TOP 08-2-500A[11].

d. The test site will follow rigorous chain-of-custody protocols as outlined in site specific procedures to minimize and mitigate sample to data errors.

4. TEST PROCEDURES.

4.1 Objectives.

Assess the efficacy of vaporous or liquid decontaminants against bacterial spores, vegetative bacterial cells, or viruses, representative of biological agents or surrogates.

4.2 Criteria and Conditions.

4.2.1 Criteria.

a. Bacterial spores. The decontaminant must demonstrate a 6-log reduction or greater from a starting challenge of $\geq 1.0 \times 10^7$ CFU per test material coupon (2 cm x 2 cm); unless otherwise defined in the test documentation.

b. Vegetative Bacteria. The decontaminant must demonstrate a 7-log reduction or greater from a starting challenge of $\geq 1.0 \times 10^8$ CFU per test material coupon (2 cm x 2 cm); unless otherwise defined in the test documentation.

c. Virus. The decontaminant must demonstrate a 7-log reduction or greater from a starting challenge of $\geq 1.0 \times 10^8$ PFU per test material coupon (2 cm x 2 cm); unless otherwise defined in the test documentation.

4.2.2 Conditions.

a. Test substrates, such as test samples, positive control samples, or negative control samples will only be used once.

b. The working stock will be assayed for viability/concentration, using the standard serial dilution and plate count procedures on the day of preparation and the day of inoculation. The titer of each preparation is confirmed through serial dilution and plating on appropriate growth media and grown under appropriate growth conditions. Plate counts and observations of colony/plaque morphology must be documented after the microbe has been incubated and grown.

c. Vaporous decontaminant generator must be capable of maintaining vapor concentration at the manufacturers claimed part per million levels.

4.3 Controls.

a. The procedures in this TOP directly compare test and control samples (room temperature controls, solutions controls, pre-neutralized controls if applicable, and negative controls). The control samples must be prepared on the same day as the test in order to increase practical confidence in the inactivation data.

b. Room temperature controls are contaminated coupons maintained at ambient laboratory conditions (22 ± 3 °C) for the duration of the test.

c. Solution controls are organisms suspended in an aqueous medium. This control represents the maximum number of organism that can be recovered since the organism is maintained suspended and never allowed to dry on a surface.

d. Pre-neutralized controls are contaminated coupons treated in an identical manner to test coupons but with a pre-neutralized decontaminant. Non-treated control coupons are used in place of test coupons for vaporous decontaminant testing. Recovery of viable organism from pre-neutralized controls on the same day(s) as the test samples provides greater confidence in the test data by eliminating time as a test variable.

e. Negative-growth controls are un-inoculated coupons, which are taken through the entire testing procedure and checked for sterility. There should be no growth in the tubes containing the negative growth control coupon. Positive growth indicates contamination and a flawed and questionable test result.

f. Additional controls may be added as necessary to address all variables in the design of experiments.

4.4 Methods and Procedures.

Not all steps in the test method(s) are applicable to all organisms and decontamination technologies; when there is a difference it will be clearly annotated.

4.4.1 Significance and Use.

a. This TOP can be used to evaluate coupon materials of any composition, insofar as the coupon can be prepared small enough to fit inside a 50-mL conical tube and/or thin enough to fit within Amicon® Ultra-15 Centrifugal Filter Units with Ultracel-100 membranes (filter units) contained in sterile 50-mL conical tubes.

b. This TOP defines procedures that are quantitative, scalable, rapid, sensitive, and safe, while minimizing labor and addressing statistical confidence.

(1) Quantitative: The total number of microorganisms per coupon is determined by dilution-plating, and all microorganisms remaining on the coupon are assayed for survival in the extraction tube to provide confidence that all microorganisms are accounted for.

(2) Statistical Confidence: Requires the use of five independent preparations of microorganisms inocula for a statistical sample size of five.

(3) Sensitivity: Determines the ability for complete detection of all culturable microorganisms inoculated on a coupon, including the microorganisms that remain attached to the coupon.

(a) The accuracy of enumeration or limit of quantification is dependent on the culturability of the microorganisms in the presence of the extraction medium appropriate for the microorganism (spore, vegetative bacteria, or virus).

(b) These media, combined with the test temperatures and conditions described herein, will generate results with a high level of practical confidence for detecting culturable microorganisms.

(4) Safety.

(a) Vaporous decontaminants: Inoculated coupons are contained within 0.2µm filter-capped 50-mL conical tubes. The 0.2 µm filter allows vaporous decontaminants to pass through while preventing escape of microorganisms, thereby providing an important level of containment when working with pathogenic strains.

(b) Liquid or solid decontaminants: Inoculated coupons are contained within filter units.

(5) Simplicity of Testing: Tests and extractions are performed in the same 50-mL conical tube, with or without filter unit, to minimize coupon handling steps.

(6) Scalable and Rapid: A maximum of 36 samples can be processed in 1 hour by two technicians; a total of 300 samples have been processed by six technicians in 5 hours. These procedures have a wide application for numerous microorganisms.

4.4.2 Hazards.

It is the responsibility of the individual user(s) of this TOP to follow all safety guidelines and to be knowledgeable about these procedures. Individual users should consult their safety authority, establish detailed safety plans and risk assessments before using this TOP. Users are strongly urged to consult the BMBL[7] and the Department of Defense Instruction (DODI) 5210.88 *Security Standard for Safeguarding BSAT*[8].

4.5 Test Procedures for Decontaminants Testing.

4.5.1 Bacterial and Viral Strains.

Specific organisms are recommended, but the choice of organism(s) should be relevant to the environment in which the decontaminant is expected to perform.

a. Pathogenic and non-pathogenic strains of Ba - Ames, Sterne, ΔSterne.

b. Acrystalliferous strains of Bt - Al Hakam, cry^- HD-1.

c. Other macrobacillus and microbacillus strains.

d. Vegetative bacteria; pathogenic and non-pathogenic – *Francisella tularensis* (Ft), Fp, *Escherichia coli*.

e. Bacteriophage/virus - MS2, Φ6.

4.5.2 Media, Culture, and Biological Agent Preparation.

a. General.

(1) Primary stocks should be maintained at ≤-60 °C in an ultra-low temperature freezer or in liquid nitrogen.

(2) It is strongly recommended that five independent preparations of each selected organism be prepared, with a minimum of three preparations required. Organisms from each independent preparation are used to prepare its corresponding independent organism inoculum, which is used to contaminate test materials.

(3) Target titer must be confirmed on the day of preparation and the day of use. The titer of each preparation is confirmed through serial dilution and plating on appropriate growth media and grown under appropriate growth conditions. Plate counts and observations of colony/plaque morphology must be documented after the microbe has been incubated and grown.

b. Bacteria. A standardized propagation method must be used and documented for the creation of the independent preparations of working stocks. Suggested methods - ASTM E3092[9], and ASTM E3178[10].

(1) Spores. Independent preparations of spores must have a titer of $\geq 1.0 \times 10^9$ CFU mL^{-1}, be ≥ 95 percent phase bright spores as determined by light microscopy and heat resistance (65 °C, 30 minutes). Coulter analysis can be used to quantify the number of spores, determine spore size, and provide additional information on spore cleanliness.

(2) Working stocks of spores must have a titer of at least one $\log_{10}$ greater than the minimum challenge concentration per test material surface (e.g., If the minimum starting challenge per coupon is 1.0×10^7 spores/coupon, then the working stock must have a minimum concentration of 1.0×10^8 spores mL^{-1}.).

(3) Vegetative Cells. Independent preparation of bacteria must have a titer of $\geq 1.0 \times 10^{10}$ CFU mL^{-1}. Coulter analysis can be used to quantify the number of cells, determine cell size, and provide additional information on cell cleanliness.

(4) Working stocks of cells must have a titer of at least one $\log_{10}$ greater than the minimum challenge concentration per test material surface.

c. Virus/Bacteriophage.

(1) Independent preparation of virus/bacteriophage must have a titer of $\geq 1.0 \times 10^{10}$ PFU mL^{-1}. If using an avian or mammalian virus, virus preparation may be propagated and assayed in the same cell line.

(2) Working stocks of virus/bacteriophage must have a titer of at least one $\log_{10}$ greater than the minimum challenge concentration per test material surface.

NOTE: Working stocks may be mixed with any number of well-characterized and standardized inorganic soil load(s) or organic soil load(s) before coupon inoculation. These loads are also known as bio burden. It must be demonstrated that the bio burden selected does not adversely affect the microorganism (i.e., it must not inhibit the organism's ability to grow and replicate). Further, the bio burden must not induce growth in the organism nor alter the physical or metabolic state of the organism.

d. On the day of preparation and use (if different from preparation date) working stocks must be verified by analysis for viability and concentration. The titer of each preparation is confirmed through serial dilution and plating on appropriate growth media and grown under appropriate growth conditions. Plate counts and observations of colony/plaque morphology must be documented.

4.5.3 Coupon Preparation and Sterilization.

a. All coupon materials must be a standardized surface area, preferably flat, 2 cm × 2 cm; however, it is understood that not all materials are easily adaptable to these size constraints.

b. Rinse coupons with 18-megaohm water.

c. Dry coupons on absorbent paper in an autoclave-safe container.

d. Autoclave temperature-insensitive coupons at 121 °C for 30 min on a wet cycle.

e. Soak materials that are temperature sensitive in pH-adjusted bleach for 10 minutes, followed by soaking in excess 90 percent ethanol for at least 15 minutes. Air dry overnight in a BSC.

f. Store sterilized coupons in sterile containers under ambient (22 ± 3 °C) laboratory conditions until use.

g. A sterility control will be used to verify coupon sterility during testing when un-inoculated coupons are taken through the entire testing procedure and checked for sterility. A negative control will be used to determine if any cross-contamination occurs during the testing process.

NOTE: Working stocks may be mixed with any number of well-characterized and standardized inorganic soil.

4.5.4 Coupon Inoculation.

a. The accurate, consistent, and reproducible application of biological agent to test material surfaces for effective quantification of decontamination efficacy is of supreme importance. Each coupon will be inoculated with a single 100 µL drop of inoculum.

b. Sterile coupons will be aseptically transferred into sterile Petri dishes inside a BSC in preparation of inoculation. Test and control coupons will be inoculated at the same time.

c. Inoculate between 12 to18 coupons at one time.

d. Allow coupons to dry uncovered, overnight in an operating BSC at ambient (22 ± 3 °C) laboratory conditions. Drying is not needed if microbes are to be tested wet.

e. Transfer inoculated coupons into pre-labeled filter-capped tubes for vaporous decontaminants or into pre-labeled filter units for liquid or solid decontaminants.

f. Store at ambient (22 ± 3 °C) laboratory conditions until used.

g. Inoculum titer must be confirmed on the day of inoculation. The titer of each preparation is confirmed through serial dilution and plating on appropriate growth media and grown under appropriate growth conditions. Plate counts and observations of colony/plaque morphology must be documented after the microbe has been incubated and grown.

h. Solution (wet) controls: These solution (wet) controls represent the maximum number of organism that can be recovered since the organism is maintained in an aqueous solution and never allowed to dry on a surface.

4.5.5 Vaporous Decontaminant Solution Control Coupon Inoculation.

a. Aseptically transfer 4.9 mL of sterile dilution buffer (e.g. 0.1 percent Tween 80, 1x phosphate buffered saline (PBS) [spores], 10 millimolar [mM] 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] + 10 percent sucrose [viruses only]) into 50-mL conical tubes with closed caps (Figure 1).

NOTE: HEPES is a zwitterionic sulfonic acid buffering agent; one of the twenty Good's buffers.

b. Transfer a single 100 µL drop of prepared inoculum to the 50-mL conical tubes containing 4.9 mL of sterile dilution buffer.

c. Inoculate between 12 to 18 solution controls at one time.

d. Cap solution control tubes and store under ambient (22 ± 3 °C) laboratory conditions until use.

Figure 1. Example of 50-mL conical tube.

4.5.6 <u>Liquid Decontaminant Solution Control Coupon Inoculation</u>.

a. Aseptically transfer 27 mL of sterile dilution buffer (e.g. 0.1 percent Tween 80, 1x PBS [spores], 10mM HEPES + 10 percent sucrose [viruses only]) into 50-mL conical tubes, and place the filter insert into the tubes (Figure 2). The 27 mL of dilution buffer prevents the solution in the filter unit from dripping through the membrane over an extended period of time, and the 100,000 molecular weight cutoff membrane keeps the organism in the filter unit.

b. Aseptically transfer 4.9 mL of sterile dilution buffer into the filter unit.

c. Transfer a single 100 µL drop of prepared inoculum to the filter unit containing 4.9 mL of sterile dilution buffer.

d. Inoculate between 12 to 18 solution controls at one time.

e. Cap solution control tubes and store under ambient (22 ± 3°C) laboratory conditions until use.

f. Spore inoculated coupons can be stored indefinitely so long as there is no loss in spore titer. The recommend storage time is no longer than 12 months.

Figure 2. Example of 50-ml conical vial with filter unit.

4.5.7 Extraction Buffers and Media.

a. The purpose of an extraction buffer is to maximize the number of organisms removed from contaminated surfaces. Selecting the most effective extraction buffer increases the practical confidence that the highest percentage of organisms have been removed from the contaminated surfaces and are available for enumeration.

b. The selection of the extraction buffer is based on maximizing the extraction efficiency without adversely influencing the number of organisms available for enumeration (i.e., if a nutrient source is a component of the extraction buffer, the extraction process must be complete in a specified time frame to ensure there is no cell division as live CFU quantification is a goal of the method).

c. Examples of extraction buffers: 10 g L^{-1} tris-buffered saline + 0.1 mol L^{-1} L-Alanine + 0.001 mol L^{-1} inosine + 0.05 percent Tween 80 for spores; 1x PBS for vegetative cells; 10 mmol L^{-1} 2-Hydroxy-3-morpholinopropanesulfonic acid + 100 mmol L^{-1} sodium chloride + 1 mmol L^{-1} calcium chloride dihydride for MS2 bacteriophage.

4.5.8 Decontamination.

a. Vaporous Decontaminants.

(1) Test and control coupons and solution controls contain $\geq 1.0 \times 10^7$ organisms. Throughout the decontamination process all environmental conditions will be monitored, controlled, and documented (temperature, RH, pressure, chemical concentration).

(2) The vaporous decontaminant will be prepared and administered as described by the product manufacturer. The use of vaporous decontaminants will most likely require placement of the test coupons and the appropriate control coupons into a cabinet for containment and controlled application of the decontaminant. Once the decontaminant exposure has occurred, the coupons will be removed and placed back into their respective tubes.

(3) Test and control samples are incubated at the appropriate test environmental conditions.

(a) Incubate control coupons at ambient laboratory conditions (22 ± 3 °C) (5 control coupons per coupon material).

(b) Incubate negative controls (uninoculated coupons) at ambient laboratory conditions (22 ± 3 °C) (1 negative control per coupon material).

(c) Incubate test solution controls (4.9 mL of dilution buffer plus 0.1 mL of organism inoculum) at the test environmental conditions with or without chemical vapor (5 test solution controls).

(d) Incubate control solution controls (4.9 mL of dilution buffer plus 0.1 mL of organism inoculum) at ambient laboratory conditions (22 ± 3 °C) (5 control solution controls).

(e) There are a total of 21 samples per coupon material. For example: five (5) coupon materials = 105 samples.

b. Liquid or solid (in a liquid form) decontaminants.

(1) There are two considerations when using a liquid decontaminant. The first is that the decontaminant and neutralizer may cause 100 percent decontamination without being able to attribute the exact percentage of decontamination to either solution. The second consideration is that it is important to determine if viable organisms are still present at some point in time after conducting a decontamination process, which requires a neutralization of the decontaminant without affecting viable organisms.

(2) This TOP uses the following procedures to select a neutralizer that stops the action of the decontaminant without killing the remaining organisms. The use of a reactive chemical compound as a decontaminant requires proper neutralization. It must be demonstrated that the selected neutralizer effectively inactivates the reactive components of the decontaminant while ensuring that the neutralizing procedure does not exert an inhibitory effect on the test organism(s)[9].

(3) A methodology study will be conducted with the selected neutralizer for a particular decontaminant. The methodology study will include information on the testing conducted to verify the efficacy of the neutralizer on the decontaminant, a description of any effects the neutralizer has on the microorganism decontaminated, and a description of any effects the neutralizer has on the materials or substrates tested. The test report must include all pertinent information from the neutralizer methodology study.

(4) Test, control coupons, and solution controls contain $\geq 1 \times 10^7$ organisms. Throughout the decontamination process all environmental conditions will be monitored, controlled, and documented (temperature, RH, and pressure).

(5) Inoculate test coupons with reactive decontaminant and incubate for the designated test conditions and time (five test coupons per coupon material). Record start and stop times.

(6) Prepare the active decontaminant as required by the manufacturer.

(7) Add 2 mL of reactive decontaminant to each sample in filter units

(8) Incubate for designated contact time at designated test conditions.

(9) After incubating for the designated contact time, 2 mL of the designated neutralizer (e.g., 5 percent sodium thiosulfate) is added to the filter units to neutralize the reactive ingredients.

(10) Incubate negative controls (uninoculated coupons) at ambient laboratory conditions (22 ± 3 °C) (1 negative control per coupon material).

(11) Incubate solution controls (4.9 mL of dilution buffer plus 0.1 mL of organism inoculum) at ambient laboratory conditions (22 ± 3 °C) (five control solution controls).

(12) Centrifugation and washing: All spore-inoculated coupons are centrifuged and washed. The solution controls and negative control coupons are not centrifuged and washed. The centrifuging and washing are described in this example:

(a) Add 10 mL of sterile, autoclaved 18-megaohm water to each filter unit to dilute the neutralized decontaminant solutions.

(b) After the addition of water, centrifuge all tubes at 3,100 × g for 10 min with maximum braking. The filter units retain all test substrates and organisms while the neutralized decontaminant is collected in the conical tubes below the filter units during centrifugation.

(c) After centrifugation, remove filter units using sterile forceps.

(d) Decant the neutralized decontaminant filtrate.

(e) Place the filter units back into the now empty conical tubes.

(f) Add 15 mL of sterile, autoclaved 18-megaohm water to each filter unit.

(g) Centrifuge at 3,100 × g for 10 min with maximum braking. The water is used to solubilize and remove potentially bacteriostatic, residual surfactants, and salt precipitates. Vortexing after the addition of water is not recommended since it adds a step and the caps can sometimes leak.

(h) After centrifugation, transfer washed filter unit coupons into new 50-mL conical tubes containing 27 mL autoclave-sterilized 18-megaohm water. The water below the filter units imparts hydrostatic pressure that inhibits extraction fluid from dripping through the membranes with minimal diffusion of the extraction buffer components through the membrane.

(i) Discard the original 50-mL conical tubes containing the filtrate.

c. Table 1 shows an example of some decontaminants and neutralizers used in developing the procedures in this TOP. The test planning process should include a rationale for the selection of neutralizers for the decontaminant(s) tested. The test report must include a description of how well the selected neutralizers performed.

TABLE 1. EXAMPLES OF DECONTAMINANTS AND ASSOCIATED NEUTRALIZERS.

DECONTAMINANT	NEUTRALIZER
2 percent calcium hypochlorite/high-test hypochlorite.	2 percent sodium thiosulfate.
Easy DECON™ 200 Decontamination Solution (DF-200).	4 percent sodium metabisulfite.
10 percent sodium hypochlorite with no hydrogen ion concentration (pH) adjustment (pH ~10).	2 percent sodium thiosulfate.
10 percent sodium hypochlorite adjusted to a pH ~7.0.	1 percent sodium thiosulfate.
Ready-to-use Spor-Klenz®.	4 percent metabisulfite.

4.5.9 Post-Decontamination Spore Extraction.

a. Vaporous decontaminant.

(1) Samples are processed in sets of 10-12 samples at a time.

(2) Add 10 mL of 1x extraction buffer to each coupon sample.

NOTE: It might be necessary to include a neutralizer in the extraction buffer for vaporous decontaminants containing a reactive compound.

(3) Add 5 mL of 2x extraction buffer to each solution (wet) control.

(4) Incubate at the appropriate conditions and for the appropriate duration for test organism(s).

(5) Vortex samples for 2 minutes on a multi-tube vortexer set at 70 percent of full speed at ambient laboratory conditions (22 ± 3 °C).

(6) Within 20 minutes of vortexing, serially dilute samples in appropriate sterile dilution buffer and plate on appropriate growth media. Directly plate 1 mL across four plates (approximately 250 µl per plate), and 0.1 mL on one plate. Transfer 0.1 mL of sample into 0.9 mL of sterile dilution buffer, serially diluting and plating on appropriate growth media at ambient laboratory conditions (22 ± 3 °C). Triplicate plating can be performed.

NOTE: Statistical precision would be improved by testing three independent samples for each microbe batch and plating each coupon independently rather than triplicate plating of a single sample. Furthermore, since triplicate plating is a measure of statistical precision it does not replace the statistical accuracy of testing 5 independent microbe batches. Statistical accuracy has greater statistical significance than statistical precision. Statistical precision from 3 independent coupons per microbe batch has greater statistical significance than triplicate plating of a single coupon. Costs and logistics will determine whether triplicate plating has value, but triplicate plating should not replace testing multiple microbe batches, because the microbe batches are fundamental to understanding reproducibility.

(7) Incubate all 50-mL conical tubes with the coupon and remaining 8.8 mL of extraction buffer at appropriate growth conditions for test organism(s). During extraction there is a period of time where the microbes do NOT replicate. That time is dependent on the microbe being tested. The samples need to be serially diluted within the time frames described in the methods so that there is no microbial replication before serial dilution and plating. For example, spores will germinate and outgrow during extraction but the serial dilution and plating should be in a time frame of ~2 h. Spore replication will not begin until 5-6 hours at room temp. The organisms remaining in the conical tube should be grown overnight to score survival of all remaining organisms on the coupon.

(8) Incubate plates at appropriate growth conditions for test organism(s).

(9) Score tubes for growth/no growth, count plates, and record data.

b. Liquid or solid decontaminant.

(1) Follow steps in Paragraph 4.5.9.a(1) through (4).

(2) Within 20 min following incubation, pipette-mix (using a P-1000 pipette) the extraction buffer in the center of the filter unit by pipetting a 1-mL volume up and down at least 5 times under ambient laboratory conditions (22 ± 3 °C).

(3) Immediately after mixing, serially dilute samples in appropriate sterile dilution buffer and plate on appropriate growth media. Directly plate 1 mL across four plates (approximately 250 µL per plate), and 0.1 mL on one plate. Transfer 0.1 mL of sample into 0.9 ml of sterile dilution buffer, serially diluting and plating on appropriate growth media at ambient laboratory conditions (22 ± 3 °C). Incubate all 50-mL conical tubes with the coupon and remaining 8.8 ml of extraction buffer at appropriate growth conditions for test organism(s).

(4) Incubate plates at appropriate growth conditions for test organism(s). Spore dilution plates should be incubated at 20 ± 5 °C for ≥24 hr. The plates can then be incubated between 15 through 37 °C for an additional 24 ± 2 hours.

(5) Score tubes for growth/no growth, count plates, and record data.

c. Scoring guidance for controls and test coupons.

(1) There should be no growth in the conical tubes containing the negative growth control coupons. Positive growth indicates contamination and a flawed and questionable test result. This control indicates that all test coupons and reagents were free of culturable biological contamination.

(2) The solution controls should be $\geq 10^6$ CFU mL^{-1} for spores and $\geq 10^7$ CFU or PFU mL^{-1} for vegetative bacteria and viruses. This will be converted to total CFU or PFU since there was 10 mL of extraction buffer.

(3) The pre-neutralized control coupons should be quantifiable titer, but the titer will vary depending on extraction efficiencies.

(4) For test coupons, if there is no growth in the 50-mL conical tube (8.8 mL of extraction buffer incubated with the coupon), no growth on plates (1.1 mL of extraction buffer was directly plated, and 0.1 mL was serially diluted and plated), then the quantity of culturable bacteria is scored as 0.0 CFU and virus is scored as 0.0 PFU. The organism population was completely inactivated as measured by this assay since there was no observed culturable growth.

(5) For test coupons, if there is growth in the 50-mL conical tube but no growth on plates, then this is recorded as positive growth. The quantity of culturable bacteria is ≥ 0.1 CFU mL^{-1} and < 1 CFU mL^{-1}, and virus is ≥ 0.1 PFU mL^{-1} and < 1 PFU mL^{-1}. The assigned quantity can range between 0.1 and 1 CFU mL^{-1} or PFU mL^{-1}. The quantity assigned in References 12, 13, 14, and 15 is 0.1 CFU mL^{-1}. No growth on the plates indicates a highly efficacious decontaminant, but growth in the 50-mL conical tube suggest that the coupon material may have protected a small number of organisms.

(6) For test coupons, all raw data for plates should be recorded. Live CFU or PFU should be recorded as organisms mL^{-1}.

5. DATA REQUIRED.

The following data will be reported in the units indicated:

a. Vaporous decontamination data should be recorded with instruments that can detect in the ranges listed below. These measurements are not intended to control the chamber environment but rather to measure the parameters during the test:

(1) Temperature (±2 °C).

(2) RH (±5 percent).

(3) Airflow speed through the chamber (±0.1 m/seconds).

(4) Elapsed time decontaminant was present (±1 minute).

(5) Contamination data for each sample area, including concentration before decontamination and residual concentration after decontamination (±10 percent CFU or PFU).

(6) Calculated log reduction (±0.1 log).

(7) All control data (10 percent CFU or PFU).

b. Liquid or solid decontamination data.

(1) Volume of decontaminant used (±0.1 mL).

(2) Decontaminant lot number.

(3) Time for decontaminant preparation (±1 minute).

(4) Time from decontaminant preparation to decontaminant application (±1 minute).

(5) Time to disseminate (±1 second).

(6) Decontaminant contact time (±1 minute).

(7) Neutralizer contact time (±1 minute).

(8) Contamination data for each sample area, including concentration before decontamination and residual concentration after decontamination (±10 percent CFU or PFU).

(9) Calculated log reduction (±0.1 log).

(10) All control data (±10 percent CFU or PFU).

c. Additional data.

(1) Laboratory temperature.

(2) Laboratory humidity.

(3) Coupon material and any material treatments.

(4) Biological agent or simulant.

(5) Name, control number/lot number, and biological agent manufacturer.

(6) Diluent used.

(7) Spore preparation yield of at least 95 percent spores (by phase-contrast microscopy) and particle size distribution using Coulter analysis.

(8) Vegetative bacterial cell preparation yield.

(9) Virus preparation yield.

(10) Genomic equivalents.

(11) Protein assay results.

(12) Microscopic results (e.g. gram stain and phase contrast).

(13) Date prepared or reconstituted.

(14) Date used.

(15) Inoculant density (10 percent CFU/mL).

(16) Dispensing device (e.g. pipettor) used.

(17) Volume of biological agent/simulant suspension applied (±1 mL).

(18) Dispensing time in seconds.

(19) Spore acceptance criteria.

(20) Sample area size (±1 cm^2).

(21) Descriptions of decontamination solutions (e.g., manufacturer, formulation, active ingredients, lot number, pot life, shelf life, and age), methods, equipment, and item-specific procedures used.

(22) Descriptions of decontaminant solution neutralizer (e.g., manufacturer, formulation, active ingredients, and lot number), methods, equipment, and item-specific procedures used.

(23) Description and photographs (or video) of any degradation of materials (e.g., corrosion, swelling) resulting from the decontaminant. A scale of reference will be included in any photographs.

(24) If applicable, a description and photographs (or video) of any special procedures performed during the decontamination process will be recorded.

(25) Any relevant safety findings as a result of testing.

6. PRESENTATION OF DATA.

a. Solution controls serve as the 100 percent recovery reference value for calculating the organism survival after decontamination. The extraction efficacy is an arithmetic calculation, not a $\log_{10}$ calculation. The extraction efficacy is calculated by dividing the total number of organisms extracted from the organism-inoculated extraction control coupons for each material type by the total number of organisms (CFU or PFU) from the solution controls for each independent preparation or sample. For simplicity and accuracy, an average extraction efficiency is calculated and recorded for each type of material (Equation 1) being tested.

$$\eta_{\text{Material}} = (\int_1^5 ec_i / sc_i)/5 \qquad \textit{(Equation 1)}$$

where:

η_{Material} = the average material extraction efficacy
ec_i = spore survival of an extraction control coupon (CFU mL^{-1})
sc_i = spore survival in the solution control (CFU mL^{-1})

b. The number of surviving organisms for each test coupon (Equation 2) and pre-neutralized coupon (if applicable in Equation 3) is corrected for extraction efficiency by dividing the number of surviving organisms by the extraction percentage to determine the total number of surviving organisms (CFU or PFU) per mL.

$$t_{(corrected\ for\ extraction\ efficacy)_i} = t_i / \eta_{\text{Material}} \qquad \textit{(Equation 2)}$$

where:

$t_{(\text{corrected for extraction efficacy})i}$ = test coupon *i*
t_i = spore survival from test coupon i (CFU mL^{-1})
η_{Material} = the average material extraction efficacy

$$pn = pn_i / \eta_{\text{Material}} \qquad \textit{(Equation 3)}$$

where:

pn(corrected for extraction efficiency)$_i$ = Pre-neutralized control coupon i
pn_i = spore survival of a pre-neutralized control coupon$_i$ (CFU mL^{-1})
$\eta_{Material}$ = the average material extraction efficacy

c. Calculate the sample mean using standard statistical techniques.

d. The organism survival of each test coupon (corrected for extraction efficiency) in organisms per mL is multiplied by 10 to account for the 10 mL total volume in each tube with extraction buffer. This will give the total number of surviving organisms (CFU or PFU) per sample. The organism survival of each control coupon and solution control organisms per mL is also multiplied by 10 to account for the 10 mL of extraction buffer.

e. Convert each number to $\log_{10}$. Since the $\log_{10}$ of 0 is negatively infinite and for simplicity, add “1” to each number before converting to $\log_{10}$.

f. Calculate the $\log_{10}$ mean (Equation 4) and $\log_{10}$ standard deviation (Equation 5) of surviving organisms for each coupon material.

$$LSM = \sum LS_i/n \qquad \textit{(Equation 4)}$$

where:

LSM = Log_{10} survival mean
LS_i = Log_{10} survival of sample i
n = number of samples

$$LSSD = \sqrt{\sum(LS_i - LSM)^2/(n-1)} \qquad \textit{(Equation 5)}$$

where:

LSSD = Log_{10} survival standard deviation
LS_i = Log_{10} survival of sample i
LSM = Log_{10} survival mean
n = number of samples

g. Calculate the log_{10} reduction mean (Equation 6) and standard deviation (Equation 7) for each coupon material.

$$LRM = LSM_{solution\ control} - LSM_{test} \qquad \text{(Equation 6)}$$

where:

LRM = Log_{10} reduction mean
$LSM_{solution\ control}$ = Log_{10} survival mean for solution controls
LSM_{test} = Log_{10} survival mean for test samples

$$LRSD = \sqrt{\frac{LSSD_{solution\ control}^{2}}{n_{solution\ control}} + LSSD_{test}^{2}/n_{test}} \qquad \text{(Equation 7)}$$

where:

$LRSD$ = Log_{10} reduction standard deviation
$LSSD$ = Log_{10} survival standard deviation
n = number of samples

h. Results may be adjusted to remove outliers in the calculations performed in Paragraphs 6.a through 6.f. References 12, 13, 14, and 15 applies Chauvenet's criterion to evaluate results with and without outliers. The maximum allowable deviation for each outlier for tests with $n=5$ is 1.645. Each individual sample is evaluated by determining the absolute value of the sample minus the sample mean and dividing that quantity by the sample standard deviation. If the result is greater than the maximum allowable deviation (Equation 8), the sample is an outlier and may be removed.

$$D_{max} \geq | x_i - x_{mean} | / \sigma \qquad \text{(Equation 8)}$$

where:

D_{max} = maximum deviation
x_i = sample i
x_{mean} = sample mean
σ = sample standard deviation

i. Data will be presented in a format to show direct comparison of pre- and post- exposure concentrations of the test coupons.

j. Data will be reported as the geometric mean to reduce the influence of outliers and to give equal weight to all experimentally derived values. The standard error of the mean will be used to calculate the error bars around the geometric mean.

k. Data Quality. A high coefficient of variation (CV) indicates greater dispersion in the variable measurements. If the CV from the replicate plate or replicate samples is too high, the data may be designated as invalid; but the data values will be reported.

l. Data Graphing. To graphically depict the raw and normalized data in terms of CFU or PFU, data bars will be shown as the geometric mean, and the geometric standard error of the mean will be used as error bars. For log reduction graphs, the geometric mean of the experimental values will be converted to $\log_{10}$.

m. Data comparison (when using normalization techniques). To compare decontamination data across materials, the mathematical techniques found in DeVries and Hamilton, *Estimating the Antimicrobial Log Reduction*: Part 1[16] and Part 2[17] will be used as follows: the experimental decontaminant's geometric mean, positive geometric standard error, and negative geometric standard error will be multiplied by a normalization value that would be required to convert the geometric mean of the water/water control to the maximum value of 5×10^8 for spores and 5×10^9 for vegetative bacteria and viruses.

APPENDIX A. BACKGROUND.

A.1. The sources of the strains used for development of this TOP were:

a. Ba ΔSterne. This strain was obtained from the Unified Culture Collection (UCC) at the USAMRIID Fort Detrick, Maryland). UCC Identifier BACI056. Ba is a Bacillus species that produces endospores that possess an exosporium; exosporium is the outermost structural layer of macrobacillus spores.

b. Bt Al Hakam. This strain was obtained from Johnathan Kiel at Brooks Air Force Base (San Antonio, Texas). Al Hakam is an acrystalliferous strain; UCC BACI229.

c. Bt cry- HD-1. This strain was obtained from Alistair Bishop. Bt cry- HD-1 is an acrystalliferous, non-insecticidal strain; Bacillus Genetic Stock Center ID4A12.

d. Fp, ATCC® 25015. Fp is a species of vegetative bacteria; this strain is a surrogate for Ft, as Ft is considered the most successful vegetative cell in former biological programs.

e. Φ6 bacteriophage. This strain was obtained from Leonard Mindich, Public Health Research Institute at New Jersey Medical School of Rutgers University, New Jersey. Φ6 is a ribonucleic acid (RNA)-enveloped virus; Pseudomonas syringae pv. phaseolicola is the host bacteria for Φ6; this strain is a surrogate for Ebola virus.

A.2. Calculations for starting concentrations are found in Table A-1

TABLE -2. EXAMPLE CALCULATIONS FOR CONTAMINATION CONCENTRATIONS

Spore Working Stock Calculations	Vegetative Cell (CFU[a]) and Virus (PFU[b]) Working Stock Calculations
Freezer stock: 1×10^{10} spores/mL	Freezer stock: 1×10^{10} CFU or PFU/mL
Working stock: Need 10 mL @ 1×10^{8} spores/mL = 1×10^{9} total spores 1×10^{9} total spores / 1×10^{10} spores/mL = 100 µL of spores + 9.900 mL of 0.1% Tween 80 = 10 mL	Working stock: Need 10 mL @ 1×10^{9} CFU or PFU/mL = 1×10^{10} total CFU or PFU 1×10^{10} total CFU or PFU / 1×10^{10} CFU or PFU/mL = 1 mL cells + 9 mL of diluent = 10 mL
Inoculation: 100 µL of 1×10^{8} spores/mL = 1×10^{7} spores/coupon	Inoculation: 100 µL of 1×10^{9} CFU or PFU/mL = 1×10^{8} CFU or PFU/coupon

[a]Colony forming units.
[b]Plaque forming units.

APPENDIX A. BACKGROUND.

A.3. A critical part of the biological decontaminant selection process in the Department of Defense (DOD), is the review of available efficacy data. Warfighter requirements necessitate that the candidate decontaminant is effective against bacterial endo-spores, particularly *Ba* spores, since it is considered the most stable and common biological agent. The availability of useful bioefficacy data is particularly challenging because of the following factors:

a. DOD requirements specifying that candidate decontaminants must be effective against both chemical and biological agents.

b. The lack of standardized biological efficacy test methods across the DOD laboratories.

c. The lack of a standard test method for quantification of efficacy against endospore-forming bacteria.

A.4. Potential decontaminant technologies currently available are loosely grouped into three categories:

a. Liquid-based decontamination sprays, which includes hypochlorite, aqueous hydrogen peroxide, aqueous ClO_2, aldehydes, germinant solutions and aqueous peracid compounds. Many of these have bacteriostatic surfactants that are part of a formula.

b. Gas and vapor-phase decontamination, which includes ethylene dioxide, ClO_2, HPV/vaporous hydrogen peroxide, methyl bromide, ozone, paraformaldehyde and HHA.

c. Energy-based decontamination, which includes UV, light and radioactive irradiation, directed energy, photochemicals, and plasma.

d. Other putative decontaminants include slurries and various dry powders.

A.5. There are currently two ASTM standards for military-relevant materials including E3092[9] for vaporous decontaminant testing, and E3178[10] for liquid decontaminant testing, both accepted in 2018. Over 150 representatives are represented within ASTM International sub-committee on anti-microbials including representatives from multiple US government agencies.

A.6. Journal articles on two tests[3, 4] published in 2018 included the Army (USAMRIID and Edgewood), Navy (Dahlgren and Navy Medical), Joint Program-Guardian, DTRA, and FDA to address the GAO report on live irradiated spores. Published data[2, 3] were recently accepted by the BBPO in May 2019 to satisfy irradiation standards requests from the GAO[1].

APPENDIX B. ADDITIONAL LABORATORY EQUIPMENT AND SUPPLIES.

B.1. APPARATUS.

a. Autoclave.

b. Shaking incubator, capable of maintaining temperature at ±2 °C with in a minimum temperature range of 25-40 °C.

c. General purpose microbiological incubator (±2 °C).

d. Phase-contrast microscope, oil immersion with magnification ≥100×.

e. Centrifuge, capable of ≥3,100xg that can hold a swinging-rotor bucket for 50-ml conical tubes.

f. Water bath, capable of maintaining temperature at ±2°C within a minimum temperature range of 50-65 °C.

g. Single-tube vortex mixer.

h. Multi-tube vortex mixer.

i. Analytical balance.

j. Ultra-low freezer, set at ≤-60 °C.

k. Stopwatch or electronic timer.

l. Manual or electronic pipettes.

m. BSC.

n. Environmental Chamber, capable of maintaining temperature ±2 °C and RH ±5 percent of target parameters; must be capable of maintaining vapor concentration.

o. Beckman Coulter Multisizer 3.

p. Bead bath, capable of maintaining temperature at ±2 °C within a minimum temperature range of 35-65 °C.

q. Tryptic soy broth (TSB).

r. Tryptic soy agar (TSA).

s. Nutrient broth (NB).

APPENDIX B. ADDITIONAL LABORATORY EQUIPMENT AND SUPPLIES.

t. Tween 80. 0.1 percent, 3 percent, and 20 percent stock solutions of Tween 80 suspended in deionized water.

u. L-alanine.

v. Inosine.

w. Sporulation broth-0.8 percent (w/v), NB or 2.5 percent (w/v) NB and salts.

x. Spore extraction buffer-pH 7.

y. Francisella growth media - 2.2 percent, mueller-hinton II cation-adjusted broth + 0.01 percent L-cysteine hydrochloric acid (HCl).

z. TSA + 0.1 percent L-cysteine HCl.

aa. E. coli - ATCC® broth6.

bb. E. coli - ATCC® agar6.

cc. MS2 Buffer6.

dd. ATCC® top agar (0.5 percent agar).

ee. 0.5 percent agar.

ff. Bacteriological agarose.

gg. HEPES.

APPENDIX C. GLOSSARY

Term	Definition
Decontaminant	A physical or chemical agent or process that inactivates pathogenic or potentially pathogenic microorganisms in or on surfaces or objects.
Endospore	A dormant, robust and non-metabolically active structure produced by certain types of bacteria from the Firmicutes phylum.
Exosporium	The outermost structural layer of macrobacillus spores including *Bacillus anthracis, Bacillus thuringiensis,* and *Bacillus cereus.*
Macrobacillus	A *Bacillus* species that produces endospores that possess an exosporium including Ba*, Bacillus thuringiensis,* and *Bacillus cereus.*
Microbacillus	A *Bacillus* species that produces endospores that do not possess an exosporium including *Bacillus subtilis* and *Bacillus atrophaeus.*
Vapor	A substance in the gas phase at a temperature lower than its critical temperature, such that it can be condensed back into a liquid by increasing the pressure on it without reducing the temperature.
Vaporous decontaminant	For the purpose of this TOP, a vaporous decontaminant can be interpreted to include gases, vapors, fogs, mists, and thermal decontaminants.
Surrogate microorganism	Microorganism that is tested to estimate responses of other microorganism(s) for which direct testing is impractical.
Virus	An infectious agent consisting of deoxyribonucleic acid (DNA) or RNA and surrounded by a protein sheath; in some cases, a membranous envelope surrounds the coat.
Envelope	A layer of host cell membrane-deprived lipid that surrounds the capsid of some viruses.
Vegetative bacteria	Bacterial cells that are metabolically active and are undergoing binary fission.

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APPENDIX D. ABBREVIATIONS.

ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection®
Ba	*Bacillus. anthracis*
BACI056	identifier for Ba ΔSterne
BACI229	identifier for Bt Al Hakam
BBPO	BSAT Biosafety Program Office
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSAT	biological select agents and toxins
BSC	biological safety cabinet
BSL	biological safety level
Bt	*Bacillus thuringiensis*
°C	degrees Celsius
CDC	Center for Disease Control
CDD	Capability Development Document
CFU	colony-forming units
ClO_2	chlorine dioxide
cm	centimeter
CV	coefficient of variation
DNA	deoxyribonucleic acid
DOD	Department of Defense
DODI	Department of Defense Instruction
DTRA	Defense Threat Reduction Agency
$eC1O_2$	electronically produced chlorine dioxide
FDA	Food and Drug Administration
Fp	Francisella philomiragia
Ft	Francisella tularensis
GAO	Government Accountability Office
HC1	hydrochloric acid
HEPA	high-efficiency particulate air
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHA	hot, humid air
ID4A12	identifier for Bt cry^{-} HD-1
mM	millimolar
MS2	methyl salicyte

APPENDIX D. ABBREVIATIONS.

NB	nutrient broth
PBS	phosphate buffered saline
PFU	plaque-forming units
QA	quality assurance
QC	quality control
RH	Relative Humidity
RNA	ribonucleic acid
TOP	Test Operations Procedure
TSA	tryptic soy agar
TSB	tryptic soy broth
UCC	Unified Culture Collection
USAMRIID	US Army Medical Research Institute for Infectious Diseases

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APPENDIX F. APPROVAL AUTHORITY.

CSTE-CI 13 April 2021

MEMORANDUM FOR

Commander, U.S. Army Operational Test Command
Director, U.S. Army Evaluation Center
Commanders, ATEC Test Centers
Technical Directors, ATEC Test Centers

SUBJECT: Test Operations Procedure 08-2-065A, Standardized Test Practices to Quantitatively Measure Liquid, Solid and Vaporous Decontaminant Efficacy on Materials Contaminated with Bacterial Spores or Other Biological Warfare Agents, Approved for Publication

1. Test Operations Procedure (TOP) 08-2-065A, Standardized Test Practices to Quantitatively Measure Liquid, Solid and Vaporous Decontaminant Efficacy on Materials Contaminated with Bacterial Spores or Other Biological Warfare Agents, has been reviewed by the U.S. Army Test and Evaluation Command (ATEC) Test Centers, the U.S. Army Operational Test Command, and the U.S. Army Evaluation Center. All comments received during the formal coordination period have been adjudicated by the preparing agency.

2. Scope of the document. This TOP describes standardized decontamination test practices and technical requirements to characterize microbes, quantitatively measure decontamination efficacy on materials contaminated with biological microbes, distinguish between static and cidal decontaminant activities, and to define practical data confidence by directly comparing controls at every step of the testing procedure.

3. This document is approved for publication and has been posted to the Reference Library of the ATEC Vision Digital Library System (VDLS). The VDLS website can be accessed at https://vdls.atc.army.mil/.

4. Comments, suggestions, or questions on this document should be addressed to U.S. Army Test and Evaluation Command (CSTE-CI), 6617 Aberdeen Boulevard-Third Floor, Aberdeen Proving Ground, MD 21005-5001; or e-mailed to usarmy.apg.atec.mbx.atec-standards@mail.mil.

ZWIEBEL.MICHAEL.J.1229197289

MICHAEL J. ZWIEBEL
Director, Directorate for Capabilities
Integration (DCI)

APPENDIX F. APPROVAL AUTHORITY.

TECMIPT Test Operations Procedure (TTOP) 08-2-065A Standardized Test Practices to Quantitatively Measure Liquid, Solid and Vaporous Decontaminant Efficacy on Materials Contaminated with Bacterial Spores or Other Biological Warfare Agents

The Contamination Mitigation Capability Area Process Action Team (CAPAT) recommends approval of the TECMIPT Test Operations Procedure (TTOP) 08-2-065A. If a representative non-concurs, a dissenting position paper will be attached.

Organization	Signature	Date
Deputy Under Secretary of the Army Test and Evaluation (DUSA-TE)	OBRIEN.SEAN.P.1230553501 Digitally signed by OBRIEN.SEAN.P.1230553501 Date: 2021.01.05 15:00:29 -05'00' Sean P. O'Brien	
Joint Program Executive Office of Chemical Biological Defense (JPEO-CBD) Test & Evaluation	No response, no dissenting position paper Joseph Rybak	
Joint Requirements Office for Chemical, Biological, Radiological and Nuclear Defense (JRO-CBRND)	[signature] MAJ Matthew S. Giffen	28 OCT 20
Joint Science and Technology Office (JSTO)	HIGH.CHRISTOPHER.JOHN.1026027671 Digitally signed by HIGH.CHRISTOPHER.JOHN.1026027671 Date: 2020.11.23 08:29:26 -05'00' Chris High	
US Army Evaluation Center (AEC)	HUGHES.JULIANE.OLSEN.1285055837 Digitally signed by HUGHES.JULIANE.OLSEN.1285055837 Date: 2021.02.23 14:28:34 -05'00' Juliane Hughes	
Operational Test and Evaluation Force (OPTEVFOR)	Philip L Engle Jr. Philip L. Engle Jr.	07 Dec 20
Air Force Operational Test and Evaluation Center (AFOTEC)	[signature] Phillip E. Hoyt, Maj, USAF, BSC	23 Nov 2020
Marine Corps Operational Test & Evaluation Activity (MCOTEA)	WADLEY.MICHAEL.CRAIG.1130810841 Digitally signed by WADLEY.MICHAEL.CRAIG.1130810841 Date: 2020.10.28 13:09:58 -04'00' Michael Wadley	
Contamination Mitigation CAPAT Co-Chair	TIENES.BRYAN.MATTHEW.1469193957 Digitally signed by TIENES.BRYAN.MATTHEW.1469193957 Date: 2020.10.16 08:57:48 -04'00' Bryan Tienes	
Contamination Mitigation CAPAT Co-Chair	BURNS.JAMES.R.III.1276086134 Digitally signed by BURNS.JAMES.R.III.1276086134 Date: 2020.12.04 11:17:56 -05'00' James Burns	

Forward comments, recommended changes, or any pertinent data, which may be of use in improving this publication to the following address: Policy and Standardization Division (CSTE-CI-P), U.S. Army Test and Evaluation Command, 6617 Aberdeen Boulevard, Aberdeen Proving Ground, Maryland 21005-5001. Technical information may be obtained from the preparing activity: Commander, West Desert Test Center, US Army Dugway Proving Ground, ATTN: TEDP-DPW, Dugway, UT 84022-5000. Additional copies can be requested through the following website: https://www.atec.army.mil/publications/documents.html, or through the Defense Technical Information Center, 8725 John J. Kingman Rd., STE 0944, Fort Belvoir, VA 22060-6218. This document is identified by the accession number (AD No.) printed on the first page.