REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
29-09-2022	Final	

4. TITLE AND SUBTITLE
Test Operations Procedure (TOP) 08-2-066B Aerosol Testing of Biological Point Detection Systems

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHORS

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Dugway Proving Ground
West Desert Test Center
(TEDP-CI)
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8. PERFORMING ORGANIZATION REPORT NUMBER
TOP 08-2-066B

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Policy and Standardization Division (CSTE-CI-P)
US Army Test and Evaluation Command
6617 Aberdeen Boulevard
Aberdeen Proving Ground, Maryland 21005-5001

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
Same as item 8

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Statement A. Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES
Defense Technical Information Center (DTIC), AD No.:
This TOP supersedes TOP 08-2-066A, dated 3 July 2013

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 provides standard procedures for designing and conducting chamber tests involving the aerosolization of biological warfare agents (BWAs) and simulants for testing biological point detectors.

15. SUBJECT TERMS
ABO – agent of biological origin; BSL – biosafety level; BWA – biological warfare agent; point detector, detector; WIBS – wideband integrated bioaerosol sensor; bacterial spores; vegetative bacteria; viruses; toxins.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	B. ABSTRACT	C. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	SAR	69	

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U.S. ARMY TEST AND EVALUATION COMMAND
TEST OPERATIONS PROCEDURE

*Test Operations Procedure 08-2-066B 29 September 2022
DTIC AD No.

AEROSOL TESTING OF BIOLOGICAL POINT DETECTION SYSTEMS

*This TOP supersedes TOP 08 2-066A, dated 3 July 2013.

1. SCOPE.

1.1 Purpose.

a. This Test Operations Procedure (TOP) provides guidelines for designing and conducting indoor and outdoor chamber tests involving the aerosolization of biological warfare agents (BWAs) and simulants, and for collecting referee data using currently available referee equipment.

b. Procedures in this TOP apply to testing with BWAs and simulants.

1.2 Application.

Test procedures in this TOP define a methodology to test the performance of biological point detection systems and their components using representatives from each of the four classes of BWAs/biological materials (bacterial spores, vegetative bacteria, viruses, and toxins).

1.3 Limitations.

a. Some of the referee equipment described in this TOP cannot be used in all aerosol test chambers because of environmental conditions or physical constraints.

b. The techniques described in this TOP for electrochemiluminescence (ECL) and polymerase chain reaction (PCR) analysis have a quantification resolution of one order of magnitude; i.e., the bacterial genome count or toxin concentration for a particular analyzed sample can be ±10 times the reported value. While common methodologies and instrumentation are described, alternative technologies may become available in the future that may be more appropriate for use.

c. Test procedures described in this TOP are for wet dissemination only.

2. FACILITIES AND INSTRUMENTATION.

2.1 Facilities.

Item	Requirement
Indoor biological chamber	Contains aerosolized biosafety level (BSL)-3 agents of biological origin (ABOs). Capable of environmental (temperature and humidity control.
Outdoor chamber	Contains aerosolized BSL-1 ABOs.
BSL-1, BSL-2, and/or BSL-3 biological laboratories	Must be constructed to ensure safe and secure storage, handling, analysis, and decontamination of microbial agents and/or simulants used for test and evaluation (T&E). A surety facility may be required for testing, depending on the strain of organism used. Required to prepare and store test quantities of biological simulant materials, to provide general support needed for work with microbial agents (e.g., to charge disseminating devices, sampler preparation), and to provide general and specialized biological analysis support for all biological agents or simulants used. Will have appropriate emergency response provisions and hazardous waste storage and disposal procedures. **NOTE**: The appropriate BSL laboratory requirements will depend on the type of organism under test.
Class II biological safety cabinet (BSC) for biological materials	Must meet the biological safety requirements for working with microbial agents and be operated according to all local standing operating procedures (SOPs). Biological surety regulations will be followed, as required.

2.2 Generic Indoor Chamber Requirements and Considerations.

a. Must be equipped with an air intake and an exhaust system that moves effluent air through high-efficiency particulate air (HEPA) filters (must remove at least 99.97 percent of particles whose diameter is equal to 0.3 μm, with the filtration efficiency increasing for particle diameters both less than and greater than 0.3 μm.

b. May be equipped with glass observation windows, access doors, and rapid transfer ports for transfer of materials (e.g., consumables and samples) in or out of the chamber.

c. An autoclave may be integrated with the chamber for sterilization of biological waste generated within the chamber. Glove ports and half-suits should be installed at select locations to

allow personnel to safely access and manipulate equipment within the chamber. The chamber may need to be equipped with electrical outlets and filtered, compressed air. Access ports for power, communication, and other cables or tubing should be considered as needed.

d. Temperature and humidity probes, as required, should be installed through the wall of the chamber on the same side as the sampling ports. Temperature and humidity control of the chamber is a primary consideration to meet testing requirements.

e. The air pressure differential between the chamber and the laboratory must be continuously monitored and adjusted to ensure that the air pressure in outer areas is always positive with respect to the pressure in inner areas.

f. Mixing fans centrally located along the base of each wall may be used to mix the aerosol inside the chamber.

2.3 Generic Outdoor Chamber Requirements and Considerations.

a. The outdoor chamber should be designed with the following capabilities: delivery of homogeneous aerosols in ambient air to the test and referee systems, characterization of cloud concentrations over time, minimization of aerosol releases into the environment, and minimization of the external meteorological conditions on internal chamber wind conditions.

b. The inside of the chamber should be equipped with multiple electrical outlets for supplying power to test systems and referee instrumentation.

c. Referee measurements in the outdoor test fixtures can be made by comparing the particle-size distributions of the generated challenge aerosol with those of the background particles to ensure that the challenge particles are larger than the background particles. The challenge aerosol particulate size must be significantly larger so that it is easy to differentiate between challenge and background particles.

2.4 Instrumentation.

Parameter	Measuring Device	Permissible Measurement Uncertainty
Liquid volume	Pipette	Varies based on volume of pipette used. Record manufacturer's stated value.
Mass	Digital scale	±0.1 milligram (mg)
Time	Digital clock	±1 second (sec)
Temperature	Digital thermometer, thermocouple, or equivalent.	±0.1 °Celsius (°C)
Relative Humidity (RH)	Digital humidity sensor, probe, or equivalent	±1 percent

Parameter	Measuring Device	Permissible Measurement Uncertainty
Wind speed	Anemometer or equivalent	±0.5 meters per second (m/sec)
Aerosol particle size	Particle sizers, such as Aerodynamic Particle Sizer (APS)™ units, slit-to-agar (STA) samplers, Wideband integrated bioaerosol sensor (WIBS), or equivalent.	±0.5 micrometer (μm)
Aerosol particle number	Particle counters, such as APS™ units, STA samplers, WIBS, or equivalent.	±10 percent number of particles per liter (L) of air
Agent-containing particles (culturable)	STA sampler	±10 percent number of particles per L of air
Biomaterial (bacteria, virus, toxin)	All glass impinger (AGI)	±10 percent number of particles per L of air
Contamination measurement	Assay-dependent	Report any contamination. Permissible error is assay-dependent.
Fluorescent particle fraction	WIBS	± 20 percent (number of fluorescent particles divided by total particles > 0.8 μm)
Test images (as required).	Digital still and video cameras	Sufficient resolution to meet test requirements.

2.5 Devices for Characterizing Challenge Aerosols in the Test Chambers.

a. STA Sampler.

(1) The STA sampler, such as a Mattson-Garvin Model 220 (Figure 1) air sampler (Mattson-Garvin, Homosassa, Florida) or equivalent, is used as the primary aerosol sampler for viable bacterial spores in the chambers. Some STA sampler models require a separate vacuum source, and other models have the vacuum source built in; the motor may or may not have more than one speed.

Figure 1. Mattson-Garvin model 220 slit-to-agar (STA) sampler.

(2) A required specification is the capability to accept a 150-millimeter (mm) diameter Petri plate containing an appropriate sterile, solid, growth medium. An air sample is drawn through a slit on the thimble mounted on top of the cover. Particles in the air sample possessing sufficient momentum impact the surface of the moist, solid, growth medium and become immobilized.

(3) After sampling, the plate is incubated and the colonies counted. Each colony represents a particle containing at least one culturable bacterium. STA samplers may be run in sequence using a vacuum sequencer.

(4) The maximum aerosol concentration should not exceed 80 agent-containing particles per liter of air (ACPLA) when using an STA sampler with a rotational speed of 1 revolution per 2 minutes; otherwise, particle over-lapping will occur on the sampler's agar surface, making quantification difficult. A time-concentration relationship can be calculated. When ambient temperatures are below 8 °C, the agar surface around the aerosol inlet may freeze.

NOTE: Vegetative bacterial cells cannot withstand impaction and cannot be accurately assayed by STA sampling. Toxins and viruses cannot be quantified with a STA sampler.

b. AGI.

(1) The AGI (Figure 2) or equivalent, is used as the primary collection tool in the chambers for BWAs that are vegetative bacteria, viruses, and toxins, as well as bacterial, viral, and toxin simulants. The AGI is used as a secondary collection for viable bacterial spores.

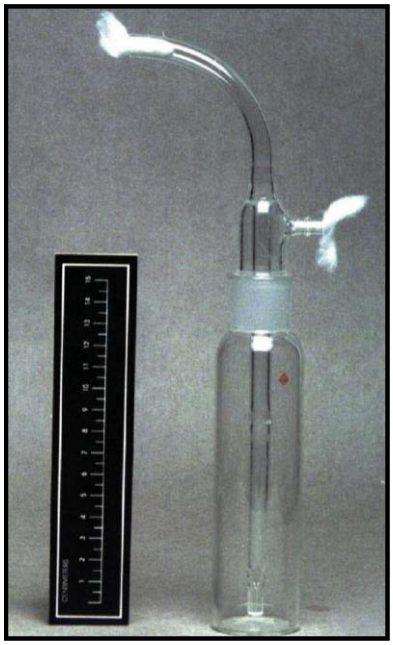

Figure 2. Example of an all-glass impinger (AGI).

(2) An AGI sample can be collected at a nominal flow rate of 12.5 liters per minute (L/min) for various time intervals. The typical duration of sample collection is 2 to 5 minutes.

(3) AGIs are typically run in sequence using a vacuum sequencer. For biological aerosol testing, 20 milliliters (mL) (per AGI) of phosphate-buffered saline (PBS) is used as the collection medium; however, other sterile diluents may be used depending on the challenge aerosol being collected. Collected samples may be assayed with various laboratory techniques, depending on the test requirements, including PCR and ECL analyses and plating or plaque assays of viable materials.

(4) There are several limitations in the use of the AGI.

(a) There is a reduction in viability of AGI-collected vegetative cells. The reduction in viability of samples is caused by physical damage to the cells during sample collection.

(b) The data are not time-resolvable, and only one data point is obtained for each sample.

(c) The liquid within the AGI is subject to freezing under some adverse combinations of sampling time and ambient temperature. The AGI may be placed in a heated rack when adverse conditions are present.

c. Aerodynamic particle sizer® (APS™).

(1) The APS™ [TSI Inc., Shoreview, Minnesota, (Figure 3)], is used to monitor particle size and concentration of particles per mL of sampled air.

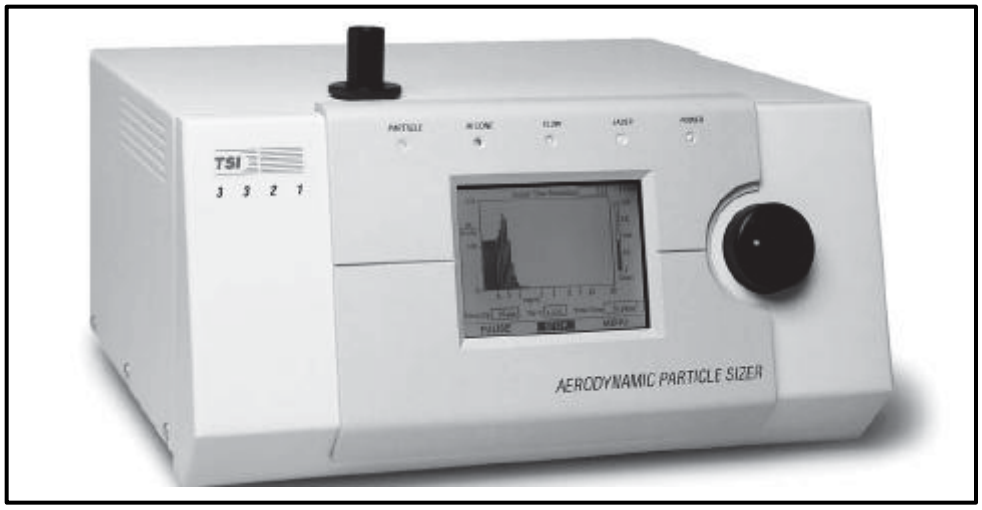

Figure 3. Aerodynamic particle sizer® (APS™) unit.

(2) Data generated by the APS™ are used to determine aerodynamic particle size and physical particle counts. The APS™ monitors particles from 0.5 up to 20 µm in aerodynamic diameter at concentrations from 1 to 1,000 particles/mL air with a coincidence error of less than 10 percent. APS™ test data are saved to an electronic file.

(3) The APS™ is typically configured to operate continuously, taking an aerosol sample every 6 seconds to determine aerosol concentration versus time. Each year, the APS™ needs to be calibrated to ensure that it is operating within manufacturer specifications.

(4) All sample lines will be isokinetic, and conductive sample tubing lengths (not to exceed 2 m) will be shortened to minimize particle loss.

d. The Wideband Integrated Bioaerosol Sensor - New Electronic Option (WIBS-NEO, Droplet Measurement Technologies, Inc., Longmont, Colorado) is an optical particle counter that measures particle concentration and particle fluorescence from for particles 0.5 - 30.0 µm. It is referred to as WIBS throughout this document. Similar to the APS™, the WIBS counts and sizes particles. However it measures true geometric particle size compared to the APS™ aerodynamic particle size measurement that depends on time of flight and density of the particle. Users should keep in mind that the WIBS will produce a different sizing result than the APS™. The WIBS is shown in Figure 4. The WIBS is operated in accordance with (IAW) its operator manual[1**] and Appendix C.

** Superscript numbers correspond to Appendix E, References.

Figure 4. Wideband Integrated Bioaerosol Sensor.

(1) The WIBS operates continuously and records a sample record for each particle that enters the inlet. A single mouse click starts the process. Several parameters can be set by the user to include fixed calibration intervals and file size limits. Data files are automatically named by date and time (e.g., 20200916153317x1.h5 is for a 15 September 20 15:33:17 data collection start time). Current real-time displays allow the user to monitor particle fluorescence and concentration versus time. WIBS Level 0 raw data must be processed off-line after a test using custom software. The vendor provides a product called the WIBS-NEO Toolkit[2] that can process the raw data using IgorPro software and convert it to particle concentration and fluorescence versus time.

(2) Particle fluorescence is determined using a reference file collected by the WIBS called the "forced trigger", or FT. This term is derived from a data collection procedure that turns off the sample pumps and forces the two Xenon flash lamps to trigger while measuring the photomultiplier tube (PMT) signal. The FT data file provides a measure of the baseline PMT noise from which the strength of particle fluorescence emission can be calculated. The FT file is typically collected for 30 seconds before and after a trial to measure the baseline PMT noise. The mean (μ_{FT}) and standard deviation (σ_{FT}) of the PMT signal is computed for each of the three fluorescence channels: A, B, and C. A particle is determined to be fluorescent if the signal strength of the emission is greater than three times the standard deviation ($3\sigma_{FT}$).

(3) The WIBS is serviced by the manufacturer by three means:

(a) Factory alignment and full unit certification.

(b) Remote assessment using TeamViewer software over a network connection that allows adjustments to detector gains and particle sizing functions.

(c) On-site service at the user's facility. On-site user confidence checks are conducted with a nebulizer system provided by the manufacturer. These methods were developmental at the date of this printing and will likely result in an additional operational procedures as they mature and gain acceptance by the T&E community.

e. The suggested quantity of standard referee instrumentation per chamber type is in Table 1.

TABLE 1. SUGGESTED QUANTITY OF STANDARD REFEREE INSTRUMENTATION.

Referee Instrument	Indoor Chamber	Outdoor Chamber
APS™	1	2
AGI	1-3	4
STA sampler (2-min)	1-3	4
WIBS	1	1

APS™ – Aerodynamic Particle Sizer®
AGI – all glass impinger
STA – slit-to-agar
WIBS – Wideband Integrated Bioaerosol Sensor

NOTE: The test officer for the test being conducted may make adjustments to these suggestions based on program needs.

f. Characterizing the time to reach concentration at the SUT.Each test setup is different, so there is no one fixed way to do it. Following is conceptual suggestion for consideration.

(1) For non-laminar air flow:

(a) Divide aerosol particle output per minute by (volume of chamber + chamber exhaust/min) to estimate particles per liter of air. Monitor with aerosol counter (e.g., APS) to confirm.

(b) Increase or decrease time in proportion to 1.a. above to achieve target concentration. Can also increase or decrease aerosol output in proportion to target concentration to keep time constant.

(2) For laminar flow:

(a) Divide aerosol output per minute by air displacement per minute. Monitor with aerosol counter.

(b) Increase or decrease aerosol output in proportion to 2.a. to achieve target concentration.

(c) Calculate time for aerosol cloud travel from source to SUT.

2.6 Generation of Near-Monodispersed Challenge Aerosols.

The Sono-Tek (Sono-Tek, Milton, New York) ultrasonic spray nozzle system (Model 8700-60) is the standard aerosol generator for chamber testing. The Sono-Tek generates near-monodispersed droplets from liquid slurries (particle suspension) which dry down to particles with the desired number mean aerodynamic diameter (NMAD). By adjusting the slurry composition, dried-down particles in the range of 2- through 6-µm NMAD can be obtained. A syringe infusion pump is employed to deliver the slurry to the nozzle and an agitator is used to prevent settling of the solid material in the slurry during delivery to the nozzle.

NOTE: Sono-Tek nozzles can also be used to generate interferent particles such as sea salt. The suspension or solution containing the interferent material can be prepared according to Equation B-1 to obtain the desired dried-down particle size.

2.7 Assay of BWA and Simulant Samples.

Standard microbiological assay procedures will be employed to quantify the number of colony forming units (CFU), plaque forming units (PFU), and mass per volume. The type of challenge material (bacteria, virus, or toxin) dictates which assay procedure and/or analytical instruments will be used.

2.8 Meteorological Measurements.

Meteorological sensors will be used in the chamber to characterize the wind speed, wind direction, temperature, and RH at the location of the system under test.

2.9 Other Test Instrumentation.

a. Automated data collection and processing systems will be used for collecting data in real time at the frequency and precision necessary for data sufficiency.

b. ECL Instrumentation and Assays. ECL is a type of immunoassay. The minitube assays for the toxin being tested will be purchased from the Joint Program Executive Office for Chemical, Biological, Radiological, and Nuclear Defense (JPEO-CBRND, Stafford Virginia) Defense Biological Product Assurance Office (DBPAO). Limit of detection and confidence levels can be requested from the DBPAO.

c. PCR Instrumentation and Assays. An example of PCR instrumentation is the Applied Biosystems Incorporated (ABI) Prism 7900HT Sequence Detection System (ABI, Carlsbad, California) or equivalent with the ABI TaqMan™ probe technology or equivalent. The ABI Prism system is a high-throughput, real-time PCR system that detects and quantifies nucleic acid sequences. Bacterial and viral samples are processed with this instrument using the TaqMan™ Detector Absolute Quantification Assay or equivalent with the 96-well plate format for PCR testing with high throughput. A bead-beating procedure is used to prepare samples of bacterial spores. Ribonucleic acid (RNA)-based viruses [such as Venezuelan equine encephalitis] are processed by reverse-transcriptase (RT)-quantitative PCR (RT-qPCR). All procedures for sample preparation for PCR (e.g., sample cleanup, deoxyribonucleic acid isolation, and PCR processing)

will be based on test site specific procedures. Resulting data are provided as genome equivalent (GE) values that can be used to calculate an aerosol concentration. For liquid samples that contain less than 5×10^3 GE/mL, the accuracy of the data is unreliable. For samples that contain greater than 5×10^3 GE/mL, the accuracy is generally within 20 percent of the actual assay concentration.

3. REQUIRED TEST CONDITIONS.

3.1 Test Planning.

A test plan (TP) should be written to document exact test procedures to be used in coordination with this TOP.

3.2 Documentation.

The following documentation should be available and planning actions taken before testing begins:

a. All pertinent current site-specific procedures shall be reviewed prior to testing.

b. Potential problem areas shall be identified by reviewing previous records, when available.

c. Development of TPs requires familiarization with the applicable test planning and requirements documents such as the Test and Evaluation Master Plan, Operational Test Agency Evaluation Plan, or Capability Development Document.

d. Safety and health issues must be given prime consideration in planning. All applicable/available safety documents such as the safety assessment report and health hazard assessments should be reviewed to determine if any safety or health issues require special test protocols.

3.3 Instrumentation Checks and Calibration.

a. Ensure all equipment and instrumentation are functioning properly.

b. Verify that all calibrated items' certificates are current and perform a pre-test instrument check to verify that drift has not occurred.

c. Ensure there is traceability between any calibrated items used and their calibration documentation, which can be accomplished by recording the unique calibration number assigned or the instrument's make, model, and serial number as part of the record.

3.4 Safety.

a. All federal, local, and site specific biological safety protocols must be followed.

b. Test personnel must be trained in the procedures to be performed. Training should be documented.

3.5 Environmental.

Site specific environmental regulations must be followed.

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

a. Each test facility's QA program will be designed to ensure that data of the required quality are obtained from each purity analysis. The data quality requirements will be established by the customer as well as by the test facility's QA/QC plan and procedures.

b. The quality of instrument data produced depends on appropriate instrument maintenance, periodic calibration, QC measures, and careful documentation of procedures. Calibration will be conducted IAW the validated calibration protocol of the test facility. In the absence of a validated protocol, calibration will be conducted as recommended by the instrument manufacturer.

c. Examples of QC measures associated with data reporting are sample preparation documentation, traceability, evaluation of analytical results, and comparison of results. QC measures will be detailed in the TP and will follow the test facility's QA/QC plan and procedures. Any problems associated with a particular sample will be noted on the appropriate log sheet or data file. All data collected must be date and time stamped.

d. Data will be independently reviewed and authenticated as required by the test facility.

e. All analysis results and calculations will be peer-reviewed and documented to ensure that random errors in transcribing data or in performing analysis are eliminated, as required by the test facility or the test program.

f. Test samples and referee samples will be kept in appropriate storage conditions until all data have been reviewed and further analytical processing of the samples is not required. Extended storage times can cause problems with viability. It is important to process any samples in a timely manner.

3.7 Chamber/Facility Preparations.

Aerosol chambers/facilities will be operated IAW the site specific SOPs.

4. TEST PROCEDURES.

4.1 Pretest.

a. The working stock of the biological material will be prepared and quality controlled. Slurries used for aerosol generation will be prepared from working stocks. The slurries will be quality controlled using the same procedures used for liquid sample analysis.

b. The chamber will be activated per appropriate operating instructions and all environmental conditions established and stabilized for at least 10 minutes.

4.2 Trial Conduct.

a. Data acquisition systems will be initiated for data collection.

b. Mixing fans required for chamber homogeneity will be activated.

c. A minimum 5 minute background collection time will be recorded using the APS™ before dissemination. This background collection time may be extended depending on the background requirements of the system under test (SUT).

d. Dissemination and sample collection time may vary depending upon the test requirements; however, a steady-state collection is recommended. For example, for a trial that requires a 5 minute aerosol generation and a 2 minute AGI sample, the AGI will be started 3 minutes after the start of aerosol dissemination to capture the steady-state aerosol concentration. If a 5 minute AGI sample is required, the AGI will be started concurrently with the start of aerosol dissemination to capture the average of the aerosol concentration. If a steady-state STA sample collection is required for a 5 minute dissemination, a 2 minute STA will be started 2 minutes before the end of aerosol generation.

e. The aerosol challenge will be disseminated IAW the TP.

f. After the end of dissemination, background levels will be sampled and recorded for 5 minutes using the APS™. This background collection time may be extended depending on the background requirements of the SUT.

g. At the end of a trial record all data into a log book and/or a data file.

h. Conduct any chamber decontamination operations as required for chamber operations to prepare for subsequent trials.

4.3 Data Handling.

See example methods in Appendices B, C, and D, as applicable. The following shall be documented:

a. Chemical receipt data:

(1) Certificate of Analysis (COA), including information on chemical purity, storage conditions, and expiration date of the standard.

(2) Chemical storage data, to include temperature logs of the storage location from receipt through use of the vial.

b. Instrumentation data: Analytical results of calibration standards and QC standard(s) as method applicable analytical results of the purity sample.

c. Final purity determination information:

(1) Report sample identification numbers and instrumentation data that were collected.

(2) Report the average purity from the triplicate run with a standard deviation.

5. DATA, DOCUMENTATION, AND RECORD REQUIREMENTS.

The data required will vary with the fixture, SUT, and test requirements, but at a minimum will include the following:

a. Characterization of the aerosol cloud, including concentration, duration, and particle size distribution.

b. Referee instrumentation used (APS™, STA sampler, AGI) and sample collection resolution will be recorded, along with sample volume, background collection time, sample interval, and collection time per sample.

c. SUT and subassembly identification numbers.

d. Complete documentation of procedures followed, sequence of events, record of observations, deviations, anomalies, and difficulties encountered.

e. Photographic documentation of representative test methods used, equipment and instrumentation systems, irregularities, component failure, or other anomalies, as required, on the SUT.

f. Environmental conditions (temperature and RH).

g. Continuous recording of critical SUT performance parameters.

h. Referee instrumentation calibration records.

i. Certification that all test and referee devices containing clocks are synchronized and recording on coordinated universal time.

j. Pre-test instrument check results.

k. Volume of deuterated solvent.

l. Storage temperature.

6. PRESENTATION OF DATA.

a. Presentation of data will be in a format that rapidly conveys whether criteria have been met, partially met, or not met. Graphs, tables, figures, and photographs will be provided as required.

b. The test results will be presented as a data package and/or in some type of report as required by the statement of work and agreement with the customer.

c. Data should be provided in the terms and units agreed upon through the data authentication group process as determined by customer coordination.

d. Data will be reported as follows:

(1) Volume of sample used for assay and volume of buffer, where applicable (e.g., aliquot of slurry, AGI samples, serial dilution, etc.) will be reported as mL.

(2) Plate counts from AGI should be reported as CFU/mL of sample or PFU/mL of sample.

(3) PCR assay should be reported as GE/mL and/or GE/L of air, depending on source of sample.

(4) ECL assay results should be reported as CFU/mL, ng/mL, and/or ng/L of air (standard curve generated with DBPAO material), depending on the source of the sample.

(5) APS™ data will be reported as ACPLA if background particles with a distinctive NMAD can be subtracted from the total particle count.

(6) STA sampler data will be reported as 4-second ACPLA values per sector (30 sectors per plate), along with moving averages for 32 seconds, 1 minute, 2 minutes, and peak dose (ACPLA).

(7) WIBS Level 3 fluorescence data for each of the three fluorescence channels (A, B, and C) will be processed and reported in units of percent fluorescence (percent F) that present the fluorescent particle fraction (FPF) multiplied by 100 (FPF × 100) in sample intervals of 10 seconds. Particle size distributions (PSD) and fluorescent particle size distributions (FPSD) will be presented in number distributions. Concentration data will be presented in units of particles per liter (ppL).

APPENDIX A. ABBREVIATIONS.

ABI	Applied Biosystems Incorporated
ABO	agents of biological origin
ABT	Ambient Breeze Tunnel
ACPLA	agent containing particles per liter of air
AD No.	Accession number
AGI	all glass impinger
ALO	agent like organism
APS™	Aerosol Particle Sizer®
ATEC	U.S. Army Test and Evaluation Command
BG	*Bacillus atrophaeus*
BSC	biological safety cabinet
BSL	biological safety level
BWA	biological warfare agent
°C	degrees Celsius
CFU	colony forming unit
COA	certificate of analysis
DBPAO	Defense Biological Product Assurance Office
DTIC	Defense Technical Information Center
ECL	electrochemiluminescence
EPSD	excited particle size distribution
FPF	fluorescent particle fraction
FPSD	fluorescent particle size distribution
FT	forced trigger
GE	genome equivalent

APPENDIX A. ABBREVIATIONS.

HEPA	high-efficiency particulate air
HDF5	Hierarchical Data Format version 5
IAW	in accordance with
JPEO-CBRND	Joint Program Executive Office for Chemical, Biological, Radiological and Nuclear Defense
L	liter
L/min	liters per minute
m/sec	meters per second
mg	milligram
mL	milliliter
mm	millimeter
MMAD	mass median aerodynamic diameter
NA	not applicable
NEO	New Electronic Option
ng	nanogram
nm	nanometer
NMAD	number mean aerodynamic diameter
PBS	phosphate buffered saline
PCR	polymerase chain reaction
percent F	percent fluorescence
PFU	plaque forming units
pg	picogram

APPENDIX A. ABBREVIATIONS.

PMT	photomultiplier tube
ppL	particles per liter
PSD	particle size distribution
QA	quality assurance
QC	quality control
qPCR	quantitative PCR
RH	relative humidity
RNA	ribonucleic acid
RT	reverse-transcriptase
sec	second
SMA	simple moving average
SOP	standing operating procedure or standard operating procedure
STA	slit-to-agar
SUT	system under test
T&E	test and evaluation
TOP	Test Operations Procedure
TP	test plan
TPSD	total particle size distribution
UV	ultraviolet
VIS	visible
WIBS	wideband integrated biological sensor
μ	mean

APPENDIX A. ABBREVIATIONS.

µm	micrometer
σ	standard deviation

APPENDIX B. PARTICLE GENERATION AND DISSEMINATION PROCEDURES WITH SLURRIES OF BIOLOGICAL MATERIAL.

a. This appendix generally addresses how particles of 2.5±0.5 µm aerodynamic diameter are produced and specifically addresses how the number of biological units (colony-forming units (CFU, plaque forming units (PFU, ng)) per particle are determined for each type of material.

b. Aerosol clouds are generated with the Sono-Tek Model 8700-60 ultrasonic spray nozzle system (Sono-Tek Corporation, Milton, New York), or equivalent. Figure B-1 provides a schematic of a Sono-Tek nozzle.

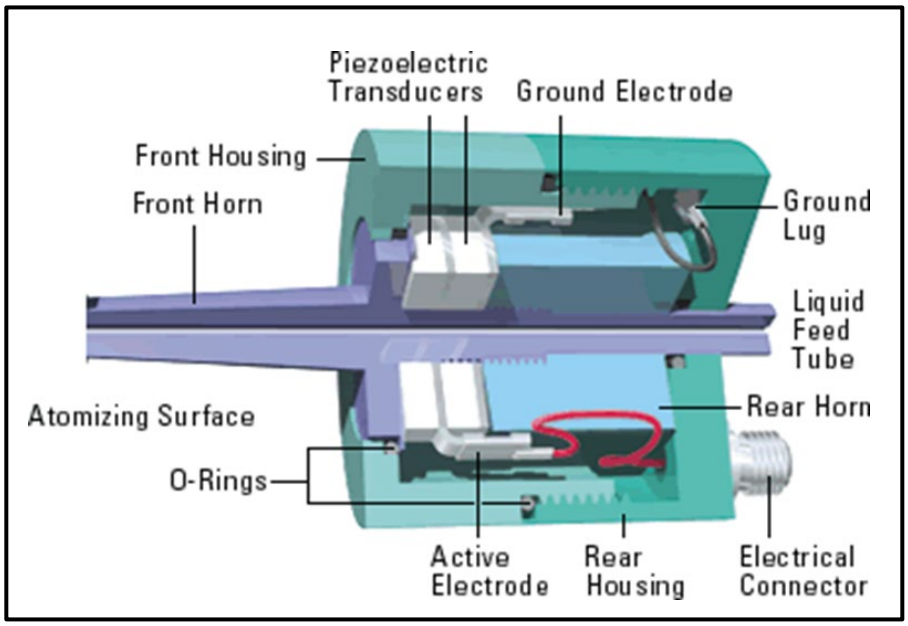

Figure B-1. Sono-Tek ultrasonic spray nozzle system model 8700-60.

c. A liquid or a liquid suspension of solid particles (slurry) is delivered to the energized nozzle. Many systems can be used to deliver the slurry to the nozzle. An infusion syringe pump is preferred because of its adjustable delivery rate and accuracy over other delivery devices, such as the peristaltic pump. The slurry concentration, the measurement of CFU/mL, and the number of particles that are disseminated in the aerosol (Appendix C, paragraph j) are interdependent. The slurry should be assayed to determine the CFU/mL, PFU/mL, or ng/mL, which can be used as a guide for calculating the quantity of each of the BWA organisms within the particles generated.

d. The Sono-Tek nozzle generates approximately 6.4×10^7 droplets per mL of liquid. The volume of 1 mL is equivalent to 1.0×10^{12} µm^3. When initially disseminated, each droplet contains both solid (BWA and salts) and liquid [water or PBS] components. The water

evaporates almost instantly, allowing the solids to coalesce. These particles are then collected using different referee systems and analyzed by various methods.

e. For the purpose of this appendix, droplets are defined as the particles generated by the Sono-Tek nozzle from the slurry. For simplicity purposes, it is assumed that dried-down particles generated from the slurry are perfectly spherical. Two common packing types will provide the optimum spherical packing density. Regardless of the type used, the maximum theoretical particle occupancy of any given space is 74 percent (geometry of close-packing spheres $\pi/3\sqrt{2} \approx 0.74048$), which leaves a void space of approximately 26 percent. In most real-world cases, however, the occupancy is lower. When spheres are randomly added to a container (for example, a tester fills a container with biological material containing droplets of 31-µm diameter) and then the spheres are compressed (the liquid is evaporated from the particles), the particles form what is known as an irregular or jammed packing configuration and can be compressed no further. This irregular packing will generally leave a void space of 36 percent, generating a value that is close to eight spores per particle in the case of *Bacillus atrophaeus* (BG). This value can then be used with agent containing particles/liter air (ACPLA, as determined with the Aerodynamic Particle Sizer® (APS™, TSI Incorporated, Shoreview, Minnesota)), to correlate ACPLA to total agent units per 2.5±0.5-µm particle for each of the biological materials used. The biological units per particle for any biological material are directly dependent on the slurry concentration, which can vary with each production batch.

f. To achieve a targeted dry particle size, the slurry must be concentrated enough to deliver the minimum solids. For example, if the target diameter size is 2.5 µm and the first dilution of the biological material stock (referred to as Slurry 1 for this example) produces 6-µm particles, as measured by the APS™, then Slurry 1 must be further diluted until the desired size is achieved. This procedure, if carried out incrementally, will allow the generation of the targeted dried-down particle size. If the initial dissemination results in a particle smaller than 2.5 µm, then a more concentrated slurry (higher solid content) is required.

g. An example of slurry preparation using biological material is the BG slurry. It is prepared by suspending a measured quantity of dry BG in a measured amount of distilled water. The resulting dried-down particle size depends mainly on the amount (volume fraction) of solids in the droplet, which is the same as the fraction of solid in the slurry. The wet particle diameter, dried-down particle diameter, and the fraction of solid in the slurry are related by the formula in Equation B-1.

$$F_v = \left(\frac{d_d}{d_w}\right)^3 \qquad \textit{(Equation B-1)}$$

where:

d_d = the diameter of the dried-down droplet (solid).
d_w = the diameter of the wet droplet.
F_v = the volume fraction of solid material in the slurry.

APPENDIX B. PARTICLE GENERATION AND DISSEMINATION PROCEDURES WITH SLURRIES OF BIOLOGICAL MATERIAL.

h. Example for Equation B-1.

(1) The d_w for the nozzles used is 31 µm, and the desired d_d is 2.5 µm. Substituting the values 31 µm and 2.5 µm into the variables in Equation B-1 yields the value for F_v of 0.000524, the volume fraction of solid material in the slurry.

(2) Once the volume fraction has been calculated, the mass of dry material can be calculated with Equation B-2.

$$m = VF_v p \qquad \textit{(Equation B-2)}$$

where:

m = the mass of dry material in grams.
V = the volume of the slurry in mL.
F_v = the volume fraction of solid material in the slurry.
p = the density of the dry material in g/mL.

(3) For example, assuming the density for BG solid is 1.0 g/cm^3, the mass of dry BG required to prepare 100 mL of slurry can be found by applying Equation B-2 yields a value of 0.0524 g form.

i. The value obtained from Equation B-2 is only the theoretical value and can be used as a starting point. In reality, the mass of dry BG required will be higher than this value because there will be some settling of solids in the delivery system, even with agitation. Therefore, the actual quantity of solids required to achieve the final dried-down particle size must be adjusted accordingly. Settling of solids in the slurry is a function of the biological material used. The settling factor for the biological material of interest can be determined experimentally.

j. Particle parameters can be individually calculated based on the above information using Equations B-3 through B-5. The biological units for slurry concentration vary depending on the type of data collected.

$$r_b = r_i c_s \qquad \textit{(Equation B-3)}$$

where:

r_b = the biological unit output rate in CFU/min, PFU/min, or ng/min.
r_i = the slurry input rate in mL/min.
c_s = the slurry concentration in biological units per mL of slurry.

APPENDIX B. PARTICLE GENERATION AND DISSEMINATION PROCEDURES WITH SLURRIES OF BIOLOGICAL MATERIAL.

$$r_d = r_i y \qquad \textit{(Equation B-4)}$$

where:

r_i = the slurry input rate in mL/min.
y = the number of droplets produced per mL of slurry.
r_d = the droplet production rate in droplets/min.

k. Equation B-5 can be used to calculate values for CFU/droplet, PFU/droplet, and ng/droplet. With the total biological units in the sample known and the biological units per particle determined, the ACPLA of the aerosol cloud can be calculated for comparison with the APS™ data (Appendix C). The calculation can also be used to convert ACPLA to CFU, PFU, or ng/L of air. **NOTE**: The biological units per particle for any biological material are directly dependent on the slurry concentration that can vary with each production batch.

$$p_b = \frac{r_b}{r_d} \qquad \textit{(Equation B-5)}$$

where:

p_b = the biological units per droplet.
r_b = the biological unit output rate in CFU/min, PFU/min or ng/min (Equation B-3)
r_d = the droplet generation rate in droplets/min (Equation B-4).

l. Non-slurry related factors that impact particle size.

(1) Relative humidity (RH). At higher RH, time required to dry down the wet particles to constant size will be longer than at lower RH. If time allowed for particle drying is limited, then the particle size measured at higher RH will appear larger than at lower RH.

(2) Distance from point of wet particle generation to point of size measurement in laminar air flow. Longer distance between the point of generation and point of measurement allows more time for drying, and hence to constant size.

(3) Time lapse from wet particle generation to time of particle size measurement in non-laminar air flow. Longer lapse time allows wet particles to dry down towards constant size.

m. All three factors above suggest in order to achieve constant particle size, sufficient time for drying is necessary.

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

TABLE LIST

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

C.1. UNITS OF MEASURE.

There are several units of measure used to express the concentration of biological material in liquid and aerosols for challenging biological detectors. These units are dependent on the class of material used to produce the slurries and aerosols. This TOP addresses four classes of biological materials: bacterial spore, vegetative bacteria, viruses, and toxins. To avoid confusion, test data should be expressed in the same units throughout the test program for each class of material. Biological units of measure used in this TOP are noted in Table C-1 and described in the following paragraphs.

TABLE C-1. BIOLOGICAL UNITS OF MEASURE

Material Class	Liquid Unit of Measure	Aerosol Unit of Measure
Bacterial Spore	Genome Equivalents (GE)/ml or colony forming units (CFU)/mL	GE/L of air or CFU/L of air and agent-containing particles/L of air (ACPLA)
Vegetative Bacteria	GE/ml or CFU/mL	GE/L of air or CFU/L air and ACPLA
Virus	GE/ml or Plaque-forming units (PFU)/mL	GE/L of air or PFU/L air and ACPLA
Toxin	Nanograms (ng)/mL	ACPLA and ng/L of air

a. Colony-Forming Units (CFU) per Unit Volume. The CFU per unit volume is used to express the concentration of bacterial spores and vegetative bacterial materials derived from electrochemiluminescence (ECL) assay or plate counts. The unit of measure is expressed in CFU/mL for liquid slurries and CFU/L of air for aerosols.

b. Plaque-Forming Units (PFU) per Unit Volume. The PFU per unit volume is used to express concentration results derived from plaque assay for viral materials derived from plaque analysis. The unit of measure is expressed in PFU/mL for liquid and PFU/L of air for aerosols.

c. Nanograms (ng) Per Unit Volume. The ng per unit volume is used to quantitate concentrations for toxin materials. The unit of measure is expressed in ng/mL for liquid slurries and ng/L of air for aerosols.

d. Genome Equivalents (GE) Per Unit Volume. The GE per unit volume is used to express concentration for bacterial spores, vegetative bacteria, and viruses derived from polymerase

chain reaction (PCR) analysis. The unit of measure is expressed in GE/mL for liquid and GE/L of air for aerosols.

e. Agent Containing Particles/L of Air (ACPLA). ACPLA is used to express the concentration of aerosol particles per L of air. ACPLA can be expressed as an average value calculated over a time interval or as an instantaneous value.

f. Aerosol Particles/L of Air without Background Particles. This value is used to express an estimate of the concentration of aerosol particles/L of air without background particles. This can also be expressed as an average value calculated over a time interval or as an instantaneous value.

g. Number Median Aerodynamic Diameter (NMAD). The NMAD, which is calculated based on the particle distribution as measured by the Aerodynamic Particle Sizer® (APS™, TSI Incorporated, Shoreview, Minnesota), provides the median aerodynamic diameter in µm of the distribution of airborne particles of a particular size bin as sampled by the APS™. Half of the number of particles in the size bin have aerodynamic diameters less than or equal to the stated NMAD bin size, and half of the number of particles have aerodynamic diameters greater than the stated NMAD bin size.

h. Mass Median Aerodynamic Diameter (MMAD). The MMAD, which is calculated based on the particle distribution as measured by the APS™, provides the median aerodynamic diameter in µm of the distribution of airborne particle mass of a particular size bin as sampled by the APS™. Half of the mass of the particles in the size bin have aerodynamic diameters less than or equal to the stated MMAD bin size, and half of the mass of all the particles have aerodynamic diameters greater than the stated MMAD bin size.

C.2. CALCULATION OF ACPLA FROM SLIT-TO-AGAR (STA) SAMPLER DATA.

a. During aerosol sampling, an agar plate is rotated by the STA turn table at a constant speed for one complete revolution. The aerosol particles carried by the STA-sampled air impact the agar plate. The agar plate is then incubated to allow the impacted bacteria to grow. Following incubation, the agar plate is divided into 30 sectors of equal area, and the number of colonies in each sector are counted and recorded. For a 2-minute STA sampling session, each of the 30 pie-shaped sectors of the agar plate represents 4 seconds of STA sampling.

b. The ACPLA for each agar sector is calculated by dividing the number of colony counts per sector by the volume of air sampled per STA sampler sector. Table C-2 contains a summary of the calculation for a single 2-min STA sampling session. Equations C-1 and C-2 show the steps to calculate ACPLA from the STA sampler data:

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

TABLE C-2. SAMPLE SECTOR COUNT DATA AND AGENT-CONTAINING PARTICLES PER LITER OF AIR (ACPLA) CALCULATION DATA FOR A 2-MIN SLIT-TO-AGAR (STA) SAMPLER WITH A SAMPLING FLOW RATE OF 28.3 L/MIN.

Sector Number	Time	Particle Count/Sector	ACPLA[a]
1	1703:00	108	57.14
2	1703:04	152	80.42
3	1703:08	141	74.60
4	1703:12	136	71.96
5	1703:16	123	65.08
6	1703:20	119	62.96
7	1703:24	130	68.78
8	1703:28	163	86.24
9	1703:32	147	77.78
10	1703:36	144	76.19
11	1703:40	156	82.54
12	1703:44	136	71.96
13	1703:48	161	85.19
14	1703:52	157	83.07
15	1703:56	151	79.89
16	1704:00	137	72.49
17	1704:04	121	64.02
18	1704:08	127	67.20
19	1704:12	137	72.49
20	1704:16	115	60.85
21	1704:20	137	72.49
22	1704:24	110	58.20
23	1704:28	102	53.97
24	1704:32	112	59.26
25	1704:36	115	60.85
26	1704:40	117	61.90
27	1704:44	128	67.72
28	1704:48	91	48.15
29	1704:52	108	57.14
30	1704:56	111	58.73

[a]Agent containing particles per liter of air.

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

$$V_{STA} = \frac{r_s t_s}{S} \qquad \textit{(Equation C-1)}$$

where:

V_{STA} = the sample volume per sector of collection media (L/sector).
r_s = the STA sampler airflow in L/min.
t_s = the sampling time in min.
S = number of sectors on the collection media.

$$\text{ACPLA} = \frac{K}{V_{STA}} \qquad \textit{(Equation C-2)}$$

where:

ACPLA = the agent containing particles per liter of air.
K = the particle count in a sector.
V_{STA} = the sample volume per sector in L/sector.

c. Given the STA sampler airflow rate of 28.3 L/min, with 2 minutes on 30 sectors, and substituting these values into Equation B-1, the sample volume is 1.89 L/sector. For the current methodology used in Whole-System Live Agent Test, the ACPLA can then be calculated with Equation C-3.

$$\text{ACPLA} = \frac{K}{1.89} \qquad \textit{(Equation C-3)}$$

where:

ACPLA = the agent containing particles per liter of air.
K = the particle count in a sector.

d. From the calculated ACPLA, three simple moving averages (SMAs) can be obtained: a 32-second moving average (over eight sectors of agar) or $SMA_{(8)}$, a 1-minute moving average or $SMA_{(15)}$ (over 15 sectors of agar), and a 2-minute moving average or $SMA_{(30)}$ (over 30 sectors of agar) (Equation B-4). Each of these three time-interval SMAs can be used to obtain smoothed plots of aerosol concentration versus time and estimates of maximum concentration. $SMA_{(8)}$, $SMA_{(15)}$, and $SMA_{(30)}$ are calculated from Equation C-4 with k set equal to 8, 15, and 30, respectively. The SMA is not defined early in the time series (Tables C-2, C-3, and C-4).

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

$$\overline{x_{l,k}} = \frac{1}{k}\sum_{i-k+1}^{l} x_l \qquad \textit{(Equation C-4)}$$

where:

$\overline{x_{l,k}}$ = the simple moving average at the i^{th} data point over k sectors (typically 8, 15, or 30 sectors for 32-second, 1-minute and 2-minute SMAs, respectively).

x_l = a data point used in the SMA.

k = the number of sectors in the SMA.

i = index of the current data point.

l = the index of a data point used in the SMA.

TABLE C-3. EXAMPLE CALCULATIONS TO OBTAIN SINGLE SLIT-TO-AGAR (STA) SAMPLER DATA 32-SECOND SIMPLE MOVING AVERAGES (SMAS)

Sample Number	Time	ACPLA[a]	8-Sector SMA Calculation	32-Second Average (ACPLA)
1	1703:00	57.14	NA[b]	NA
2	1703:04	80.42	NA	NA
3	1703:08	74.60	NA	NA
4	1703:12	71.96	NA	NA
5	1703:16	65.08	NA	NA
6	1703:20	62.96	NA	NA
7	1703:24	68.78	NA	NA
8	1703:28	86.24	(Addition of Samples 1 through 8)/8	70.90
9	1703:32	77.78	(Addition of Samples 2 through 9)/8	73.48
10	1703:36	76.19	(Addition of Samples 3 through 10)/8	72.95
11	1703:40	82.54	(Addition of Samples 4 through 11)/8	73.94
12	1703:44	71.96	(Addition of Samples 5 through 12)/8	73.94
13	1703:48	85.19	(Addition of Samples 6 through 13)/8	76.46

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

TABLE C-3. CONTINUED

Sample Number	Time	ACPLA[a]	8-Sector SMA Calculation	32-Second Average (ACPLA)
14	1703:52	83.07	(Addition of Samples 7 through 14)/8	78.97
15	1703:56	79.89	(Addition of Samples 8 through 15)/8	80.36
16	1704:00	72.49	(Addition of Samples 9 through 16)/8	78.64
17	1704:04	64.02	(Addition of Samples 10 through 17)/8	76.92
18	1704:08	67.20	(Addition of Samples 11 through 18)/8	75.79
19	1704:12	72.49	(Addition of Samples 12 through 19)/8	74.54
20	1704:16	60.85	(Addition of Samples 13 through 20)/8	73.15
21	1704:20	72.49	(Addition of Samples 14 through 21)/8	71.56
22	1704:24	58.20	(Addition of Samples 15 through 22)/8	68.45
23	1704:28	53.97	(Addition of Samples 16 through 23)/8	65.21
24	1704:32	59.26	(Addition of Samples 17 through 24)/8	63.56
25	1704:36	60.85	(Addition of Samples 18 through 25)/8	63.16
26	1704:40	61.90	(Addition of Samples 19 through 26)/8	62.50
27	1704:44	67.72	(Addition of Samples 20 through 27)/8	61.90
28	1704:48	48.15	(Addition of Samples 21 through 28)/8	60.32
29	1704:52	57.14	(Addition of Samples 22 through 29)/8	58.40
30	1704:56	58.73	(Addition of Samples 23 through 30)/8	58.47

[a]Agent containing particles per liter of air.
[b]Not applicable.

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

TABLE C-4. EXAMPLE CALCULATIONS TO OBTAIN SINGLE STA SAMPLER DATA 1-MINUTE SIMPLE MOVING AVERAGES.

Sample Number	Time	ACPLA[a]	15-Sector Calculation	1-minute Average (ACPLA)
1	1703:00	57.14	Not applicable (NA)	NA
2	1703:04	80.42	NA	NA
3	1703:08	74.60	NA	NA
4	1703:12	71.96	NA	NA
5	1703:16	65.08	NA	NA
6	1703:20	62.96	NA	NA
7	1703:24	68.78	NA	NA
8	1703:28	86.24	NA	NA
9	1703:32	77.78	NA	NA
10	1703:36	76.19	NA	NA
11	1703:40	82.54	NA	NA
12	1703:44	71.96	NA	NA
13	1703:48	85.19	NA	NA
14	1703:52	83.07	NA	NA
15	1703:56	79.89	(Addition of Samples 1 through 15)/15	74.92
16	1704:00	72.49	(Addition of Samples 2 through 16)/15	75.94
17	1704:04	64.02	(Addition of Samples 3 through 17)/15	74.85
18	1704:08	67.20	(Addition of Samples 4 through 18)/15	74.36
19	1704:12	72.49	(Addition of Samples 5 through 19)/15	74.39
20	1704:16	60.85	(Addition of Samples 6 through 20)/15	74.11

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

TABLE C-4. CONTINUED

Sample Number	Time	ACPLA[a]	15-Sector Calculation	1-minute Average (ACPLA)
21	1704:20	72.49	(Addition of Samples 7 through 21)/15	74.74
22	1704:24	58.20	(Addition of Samples 8 through 22)/15	74.04
23	1704:28	53.97	(Addition of Samples 9 through 23)/15	71.89
24	1704:32	59.26	(Addition of Samples 10 through 24)/15	70.65
25	1704:36	60.85	(Addition of Samples 11 through 25)/15	69.63
26	1704:40	61.90	(Addition of Samples 12 through 26)/15	68.25
27	1704:44	67.72	(Addition of Samples 13 through 27)/15	67.97
28	1704:48	48.15	(Addition of Samples 14 through 28)/15	65.50
29	1704:52	57.14	(Addition of Samples 15 through 29)/15	63.77
30	1704:56	58.73	(Addition of Samples 16 through 30)/15	62.36

[a]Agent containing particles per liter of air.

e. For multiple STA samplers, the 32-second SMA, 1-minute SMA, 2-minute SMA, and corresponding peaks are calculated in a similar fashion (Equation C-5).

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

$$X_k = max_i \overline{x_{l,k}} \qquad \textit{(Equation C-5)}$$

where:

X_k = the maximum of the moving average over k data points (8, 15 or 30 data points).

$\overline{x_{l,k}}$ = the simple moving average at the i^{th} data point over k sectors (typically 8, 15, or 30 sectors for 32-sec and 1- and 2-min SMAs) from Equation C.4.

k = the number of data points in the moving average.

i = the index of a data point used in the moving average

TABLE C-5. EXAMPLE CALCULATIONS TO OBTAIN SINGLE SLIT-TO-AGAR (STA) SAMPLER DATA 2-MIN SIMPLE MOVING AVERAGES (SMAS)

Sample Number	Time	ACPLA[a]	15-Sector Calculation	1-minute Average (ACPLA)
1	1703:00	57.14	Not applicable (NA)	NA
2	1703:04	80.42	NA	NA
3	1703:08	74.60	NA	NA
4	1703:12	71.96	NA	NA
5	1703:16	65.08	NA	NA
6	1703:20	62.96	NA	NA
7	1703:24	68.78	NA	NA
8	1703:28	86.24	NA	NA
9	1703:32	77.78	NA	NA
10	1703:36	76.19	NA	NA
11	1703:40	82.54	NA	NA
12	1703:44	71.96	NA	NA
13	1703:48	85.19	NA	NA
14	1703:52	83.07	NA	NA
15	1703:56	79.89	NA	NA
16	1704:00	72.49	NA	NA
17	1704:04	64.02	NA	NA
18	1704:08	67.20	NA	NA
19	1704:12	72.49	NA	NA
20	1704:16	60.85	NA	NA
21	1704:20	72.49	NA	NA
22	1704:24	58.20	NA	NA

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

TABLE C-5. CONTINUED

Sample Number	Time	ACPLA[a]	15-Sector Calculation	1-minute Average (ACPLA)
23	1704:28	53.97	NA	NA
24	1704:32	59.26	NA	NA
25	1704:36	60.85	NA	NA
26	1704:40	61.90	NA	NA
27	1704:44	67.72	NA	NA
28	1704:48	48.15	NA	NA
29	1704:52	57.14	NA	NA
30	1704:56	58.73	(Addition of Samples 1 through 30)/30	68.64

[a]Agent containing particles per liter of air.

NOTE: Notice the first average value starts at sample number 30.

C.3. WIBS.

Data generated by the WIBS are used to determine the number of fluorescent particles, N_F, in an aerosol challenge presented to a SUT relative to the total number of aerosol particles that are collected, N_T. The critical parameter of interest for testing is the fluorescent particle fraction ($FPF = N_F / N_T$). There are three independent N_F measurements in each of three fluorescence channels using two ultraviolet (UV) excitation wavelengths (280 nm and 370 nm) and two emission bands [UV: 310-400 nm, and visible (VIS): 420-650 nm]. The 370 nm source saturates the UV detector, so that channel is not usable. The fluorescence channels are referred to as Channels A, B, and C as illustrated in Table C-6. The WIBS generates data for each particle that enters the sample inlet in the particle range of 0.5 - 30.0 µm, recording its size and fluorescence magnitude for the three fluorescence channels. Data files are recorded in an industry-standard Hierarchical Data Format version 5 (HDF5). WIBS performance specifications and data file fields are described in detail in the operator manual[3].

TABLE C-6. WIBS FLUORESCENCE CHANNELS A, B AND C

Excitation Wavelength	Emission Band	
	310 – 400 nm	420 – 650 nm
280 nm	Channel A	Channel B
370 nm	Not applicable	Channel C

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

C.4. CALCULATION OF CFU/L AND PFU/L OF AIR FROM PLATE DATA.

a. Bacterial and viral material grown on plates are counted using the same procedure, except that either bacterial colonies or viral plaques are counted.

(1) Plate counts from bacterial spores are determined by taking three aliquots (usually 0.2 mL each) from an AGI sample, or SUT sample. Each 0.2-mL sample is spread on an agar plate, the plate is incubated, and the number of colonies on each plate are counted. If the bacterial growth appears to be too numerous to count, appropriate dilutions must be made, and the diluted samples plated. The total counts are recorded on a spreadsheet.

(2) Plaque counts from virus materials are determined by taking three 0.2-mL samples from an AGI sample, or SUT sample, spreading the samples on three plates containing host cells, incubating the plates, and counting the number of plaques on each plate. If the plaques appear too numerous to count, appropriate dilutions are made and diluted samples are re-plated. The total counts are recorded on a spreadsheet.

b. CFU/mL and PFU/mL are calculated by multiplying the average plate count by the sample dilution factor (if applicable) and by a scaling factor to scale the result to a 1-mL sample (Equation C-6).

$$c = \frac{k_p f_d}{V_s} \qquad \textit{(Equation C-6)}$$

where:

c = the volumetric concentration expressed in biological units per mL.
k_p = the average plate count.
f_d = the dilution factor.
V_s = the sample volume in mL (usually 0.1 or 0.2 mL).

c. An average liquid volumetric concentration in biological units per mL (CFU/mL or PFU/mL) is obtained by adding the three concentration values and dividing the resulting value by three (Equation C-7). The total biological units are calculated by multiplying the average liquid volumetric concentration by the total sample volume (in mL). The total CFU is converted to CFU/L air by dividing the total CFU by the sampling time (in minutes) multiplied by the system air-flow rate (L/minute). Tables C-7 and C-8 provide an example for calculating the CFU/L and PFU/L air from a 5-minute AGI sample with a total volume of 16.3 mL. Each of the triplicate samples is 200 µL. The AGI airflow rate is 12.5 L/min.

APPENDIX C. REFEREE INSTRUMENTATION DATA REDUCTION AND CONVERSION.

$$\overline{c_l} = \frac{1}{n}\sum_{j=1}^{n} c_j \qquad \textit{(Equation C-7)}$$

where:

c_j = the liquid volumetric concentration in each sample in biological units per mL.
$\overline{c_l}$ = the average liquid volumetric concentration in biological units per mL.
j = the index of each sample.
n = the number of samples.

5. CALCULATING THE AIR CHALLENGE CONCENTRATION BASED ON THE LIQUID SAMPLE CONCENTRATION.

a. To calculate the air challenge concentration based on the liquid sample concentration, first calculate the total GE, CFU, PFU, or ng contained in the liquid sample (Equation C-8).

$$T = \overline{c_l} V_s \qquad \textit{(Equation C-8)}$$

where:

T = the total biological units in the liquid impinger sample (GE, CFU, PFU or ng).
$\overline{c_l}$ = the average concentration (Equation C-7) of a liquid sample in biological units/mL.
V_s = the sample volume in mL.

b. The air concentration can then be obtained by dividing the liquid sample concentration from Equation C-8, by the sampling time multiplied by the sampling airflow rate (Equation C-9).

$$c_a = \frac{T}{t_s r_s} \qquad \textit{(Equation C-9)}$$

where:

c_a = the air concentration in biological units/L of air.

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T = the total biological units in the liquid impinger sample (GE, CFU, PFU, or ng) from Equation C-8.
t_s = the sampling time in minutes.
r_s = the sampling airflow rate in L/min.

C.6. CALCULATION OF AEROSOL CONCENTRATION IN BIOLOGICAL UNITS/L AIR FROM AEROSOL CONCENTRATION IN ACPLA.

a. For an aerosol generated from a slurry, the air concentration in CFU/L air, PFU/L air, or ng/L air, can be calculated using Equation C-10 to produce data as found in Tables C-7 or C-8. Three assumptions are made in this calculation:

(1) The aerosol droplets are spherical.

(2) Each slurry droplet dries to form one solid aerosol particle.

(3) The droplets are near-monodispersed.

$$c_a = ACPLA \times V_d \times c_s = ACPLA \times 1.56 \times 10^{-8} \times c_s \qquad \textit{(Equation C-10)}$$

where:

c_a = the air concentration in biological units/L of air (Equation B-9).
ACPLA = the aerosol concentration in agent-containing particles per liter of air.
V_d = the aerosol median droplet volume in mL.
c_s = the slurry concentration in biological units/mL.

TABLE C-7. EXAMPLE OF CALCULATION OF COLONY-FORMING UNITS (CFU) PER LITER OF AIR (CFU/L AIR) FROM PLATE DATA

Sample Number	Colony Counts per plate	Dilution Factor	CFU/mL[a]	Average CFU/mL	Volume of AGI[b] Sample (mL)	Total CFU	AGI Flow Rate (L air/min)	Sample Time (min)	CFU/L air
1	46	10	$46 \times 10 \times 5 = 2.30 \times 10^3$	2.23×10^3	16.3c	$2.23 \times 10^3 \times 16.3 = 3.63 \times 10^4$	12.5	5	$\frac{3.63 \times 10^4}{(12.5 \times 5)} = 5.81 \times 10^2$
2	45	10	$45 \times 10 \times 5 = 2.25 \times 10^3$						
3	43	10	$43 \times 10 \times 5 = 2.15 \times 10^3$						

[a]CFU/mL – colony-forming units (CFU)/mL (determined by plating 0.2 mL of liquid bacterial sample, incubating the plate, and counting any growth).
[b]The volume of the all-glass impinger (AGI) sample is 16.3 mL in this table; however, this is an example volume, and the actual volume may vary depending upon test requirements.

TABLE C-8. EXAMPLE OF CALCULATION OF PLAQUE-FORMING UNITS (PFU) PER LITER OF AIR (PFU/L AIR) FROM PLAQUE DATA

Sample Number	Colony Counts per Plate	Dilution Factor	PFU/mL[a]	Average PFU/mL	Volume of AGI Sample (mL)[b]	Total PFU	AGI Flow Rate (L air/ min)	Sample Time (min)	PFU/L air
1	78	1	$78 \times 1 \times 5 = 3.90 \times 10^2$	4.70×10^2	16.6	$4.70 \times 10^2 \times 16.6 = 7.80 \times 10^3$	12.5	5	$\frac{7.80 \times 10^3}{(12.5 \times 5)} = 1.25 \times 10^2$
2	103	1	$103 \times 1 \times 5 = 5.15 \times 10^2$						
3	101	1	$101 \times 1 \times 5 = 5.05 \times 10^2$						

[a]CFU/mL - colony-forming units (CFU)/mL [determined by plating 0.2 mL of liquid bacterial sample, incubating the plate, and counting any growth].

[b]The volume of the all-glass impinger (AGI) sample is 16.3 mL in this table; however, this is an example volume, and the actual volume may vary depending upon test requirements.

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TABLE C-9. AIRFLOW RATE AND COMMONLY USED SAMPLING TIMES FOR DIFFERENT REFEREE SYSTEMS.

System[a]	Sampling Airflow Rate (L/min)	Sampling Time (minutes)
STA sampler	28.3	2.0
AGI	12.5	2.0 or 5.0

[a]STA – slit-to-agar; AGI – all-glass impinger.

b. For example, for a nozzle producing an aerosol with a mean droplet diameter of 31 µm, disseminating the slurry with a concentration of 5.0×10^8 CFU/mL, and achieving an aerosol concentration of 100 ACPLA, the CFU/L air can be calculated using Equation C-11.

$$c_a = 100 * 1.56 \times 10^{-8} * 5.0 \times 10^8 = 780 \text{ CFU/L} \qquad \textit{(Equation C-11)}$$

where:

c_a = the air concentration in biological units/L of air (Equation C-9).

C.7. CALCULATING AEROSOL PARTICLE CONCENTRATION FROM GE OF AGENT - LIKE ORGANISM (ALO) OR SIMULANT.

a. This critical computation allows testers to correlate values from BWA with ALO or simulant aerosolization. There are inherent errors that force careful consideration when making such a comparison and when using the values generated. There are two instances where use of this conversion is essential:

(1) When ACPLA values cannot be directly calculated from known dissemination and operating parameters such as dissemination rates, number of particles generated, and chamber volumetric flow rates.

(2) During field disseminations when concentrations are below natural background levels.

b. Calculating ACPLA from GE/mL is done through the use of a conversion factor. The conversion factor can be found for live materials using the ratio of the stock culture CFU/mL or

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PFU/mL to GE/mL, and for inactivated materials by using the ratio of the stock culture CFU/mL or PFU/mL to the irradiated GE/mL. The concentration of the stock culture should be determined by PCR analysis and plate count or plaque assay as soon as possible after production. This will provide the most accurate ratio of live to genetic quantities. For inactivated biological ALO or simulants, the PCR analysis value should also be determined after the ALO/simulant has been killed or inactivated. This procedure provides data to determine the amount of genetic material destroyed in the deactivation process. A typical PCR analysis result from a field sample is given in GE/mL. ACPLA from GE/mL can be calculated using Equation C-12.

$$ACPLA = \frac{c_g V_s f_c}{V_a \rho_b} \qquad \textit{(Equation C-12)}$$

where:

c_g = the concentration of ALO or simulant in the liquid sample in GE/mL.
V_s = the liquid sample volume in mL.
f_c = the conversion factor in CFU/mL or PFU/mL per GE.
V_a = the volume of air sampled in L.
ρ_b = the average number of biological units per mL.

C.8. TIME-SERIES AVERAGE APS™ PARTICLE CONCENTRATION.

a. For an environment with negligible background noise, the time-series average concentration of aerosol particles per liter of air can be estimated for different time lengths using Equation C-4, applied to the number of particles collected at each time step. The number of particles collected over a time period is the sum of overall size bins (Equation C-13).

$$b_\tau = \sum_{i=1}^{N} b_{\tau,i} \qquad \textit{(Equation C-13)}$$

where:

b_τ = the number of particles collected at each time step.
$b_{\tau,i}$ = the number of particles in each size bin at each time step.
i = the bin index, each bin corresponds to a particle size.
τ = the index of each time step.
N = the number of particle size bins.

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C.9. CALCULATIONS OF APS™ NMAD.

a. The NMAD value is calculated by using data collected after the disseminated aerosol concentration has reached a steady state. In a controlled chamber environment a steady-state aerosol concentration is typically reached after three chamber-volume air exchanges. A good estimation of the NMAD value can be obtained by sampling an aerosol with an APS™ for 2 minutes after the aerosol has reached a steady-state concentration.

b. In general, the number of particles contained in the first APS™ size bin (<0.523 μm) are not considered for the NMAD calculation because:

(1) Particles less than 0.523 μm in diameter are not representative of typical BWA threats which are at least 1 μm in diameter. Particles in this bin are mostly non-cellular and interfere with the biological picture of the data.

(2) The aerosol generated by a Sono-Tek nozzle produces a narrow range of particles distributed around the desired NMAD bin value with very small number of particles below 1 μm in diameter.

(3) Particles less than 0.523 μm are mainly chamber background particles.

c. Chamber background particles are the aerosolized particles that occur in the chamber without the intentional dissemination of an aerosol. Figure C-1 provides a typical particle distribution for a 2.5-μm aerosol cloud in a clean chamber environment generated with Sono-Tek nozzle (Model 8700-60) producing 31-μm droplets. The particle distribution in Figure C-1 was obtained by calculating for each APS™ bin the average percentage number of particles from the total number of particles observed during 7 minutes of steady-state aerosol dissemination from 145 trials.

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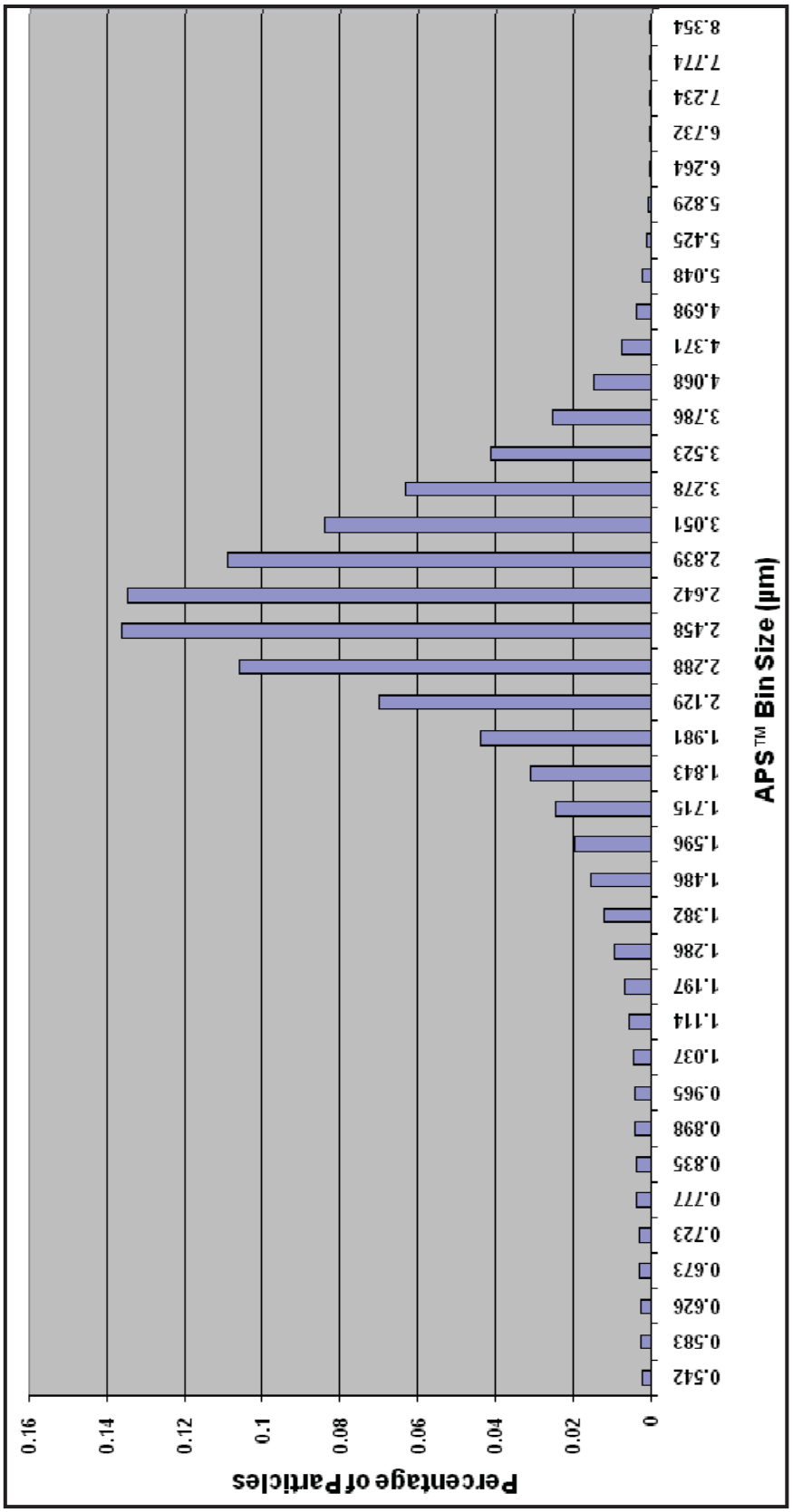

Figure C-1. Average particle distribution for a 2.5-µm number mean aerodynamic diameter (NMAD) aerosol.

NOTE: The aerosol was generated with a Sono-Tek nozzle (Sono-Tek Corporation, Milton, New York) producing 31-µm droplets. The sample of aerosol was collected and analyzed by an Aerodynamic Particle Sizer® (APS™, TSI Inc., Shoreview,Minnesota).

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d. For an environment with negligible background noise, the NMAD can be calculated by finding the bin that contains the particles in the fiftieth percentile. The total number of particles must be calculated (Equation C-14).

$$N_t = \sum_i \sum_\tau b_{\tau,i} \qquad \textit{(Equation C-14)}$$

where:

N_t = the total number of particles collected over the duration of the trial.
$b_{\tau,i}$ = the number of particles in each size bin at each time step.
i = the bin index, each bin corresponds to a particle size.
τ = the index of each time step.

e. Percentiles would be calculated using Equation C-15. The NMAD is the smallest particle size bin where p_k is greater than 50 percent.

$$p_k = \frac{1}{N_t} \sum_{i-1}^{k} b_i \qquad \textit{(Equation C-15)}$$

where:

p_k = the percentile of the bin with index k.
N_t = the total number of particles collected over the duration of the trial from Equation C-14.
b_i = the number of particles in each bin through the duration of the trial from Equation C-16.
i = the bin index, each bin corresponds to a particle size.
k = the number of data points in the moving average.

$$b_i = \sum_{\tau=0}^{\tau_{end}} b_{\tau,i} \qquad \textit{(Equation C-16)}$$

where:

b_i = the total number of particles in each bin through the duration of the trial.
τ = the index of each time step.
τ_{end} = the index of the last time step (it is also the total number of time steps).
$b_{\tau,i}$ = the number of particles in each size bin at each time step.

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C.10. CALCULATIONS OF APS™ MMAD.

a. The MMAD value is calculated by using data after the disseminated aerosol concentration has reached a steady state. In a controlled chamber environment, a steady-state aerosol concentration is typically reached after three chamber volume air exchanges. A good estimation of the MMAD value can be obtained by sampling an aerosol with an APS™ for 2 minutes after the aerosol has reached a steady-state concentration.

b. In general, the number of particles contained in the first APS™ bin (<0.523 µm) are not considered for the MMAD calculation because:

(1) Particles in the <0.523-µm bin are not BWA threat representative;

(2) The aerosol generated by a Sono-Tek nozzle produces a narrow range of particles centered about the desired M bin value with a very small number of particles below 1 µm

(3) Particles in the 0.523-µm bin are mainly chamber background particles. Chamber background particles are the concentration of aerosolized particles occurring in a chamber without the explicit or intentional dissemination of an aerosol. For an environment with negligible background, the MMAD can be calculated by summing all the particles in each size bin (Equation C-16), then calculating the mass for each bin (Equation C-17); afterwards, the total mass is calculated (Equation C-18), and finally, the percentiles of each bin are calculated (Equation C-19) and the MMAD found.

$$m_i = \frac{\pi}{6} d_i^3 b_i \rho \qquad \textit{(Equation C-17)}$$

where:

m_i = the mass in each size bin in picograms (pg).
d_i = the aerodynamic diameter of each size bin in µm.
b_i = the number of particles in each size bin through the duration of the trial (Equation C-16).
ρ = the density of the dry aerosol particles (formed from the slurry) in g/mL.

$$m_{tot} = \sum_i m_i \qquad \textit{(Equation C-18)}$$

where:

m_i = the mass in each size bin in pg (Equation C-17).
m_{tot} = the total mass of all particles in pg.

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i = the bin index, each bin corresponds to a particle size.

$$p_k = \frac{1}{m_{tot}} \sum_{i=1}^{k} m_i \qquad \textit{(Equation C-19)}$$

where:

p_k = the mass percentile of the bin with index k.
m_{tot} = the total mass in pg (calculated in Equation B-18).
m_i = the mass in each size bin in pg (calculated in Equation B-17).
i = the index of each particle size bin.
k = the number of data points in the moving average.

c. The MMAD is the aerodynamic diameter of the first bin with a percentile greater than 50 percent.

C.11. ESTIMATION OF AEROSOL CONCENTRATION FROM ATOMIZED SLURRY IN THE AMBIENT BREEZE TUNNEL (ABT) CHAMBER.

a. An estimate of the average aerosol concentration in particles/L of air in the ABTcan be obtained if the slurry dissemination feed rate and the size of the aerosol droplets are known (Equation C-20). The aerosol droplet volume is calculated in Equation C-21.

$$\overline{c_p} = \frac{S}{60000AWV_d} \qquad \textit{(Equation C-20)}$$

where:

c_p = the average aerosol particle concentration in particles per liter of air.
S = the slurry dissemination rate in mL/min.
A = the cross-sectional area of the breeze tunnel in m^2 (33.17 m^2 in the ABT).
W = average breeze tunnel wind speed in m/sec.
V_d = the aerosol median droplet volume in mL (Equation C-21).

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$$V_d = \frac{\pi d^3}{6} \qquad \textit{(Equation C-21)}$$

where:

V_d = the aerosol median droplet volume in mL.
d = the median diameter in cm.

b. An example is given in Equation C-22 using Equation C-21. For a nozzle producing an aerosol with a mean droplet diameter of 31 µm, the volume per droplet of disseminated slurry (V_d) is calculated in Equation C-22.

$$V_d = \frac{\pi d^3}{6} = \frac{\Pi(31 \times 10^{-4} cm)^3}{6} = 1.56 \times 10^{-8} mL \qquad \textit{(Equation C-22)}$$

c. Example for Equation C-20. The ABT has a cross-sectional area of 33.17 m^2. If an aerosol is generated in the ABT at a feed rate of 4 mL/min and with an average ABT windspeed of 1 m/sec, the estimated average aerosol concentration (C) is calculated in Equation C-23.

$$C = \frac{S}{60000AWV_d} = \frac{4}{60000 \times 33.17 \times 1 \times 1.56 \times 10^{-8}} = 129\, particle/L \qquad \textit{(Equation C-23)}$$

where:

C = average aerosol concentration
S = the slurry dissemination rate in mL/min.
A = the cross-sectional area of the of the breeze tunnel in m^2 (33.17 m^2 in the ABT).
W = the average breeze tunnel wind speed in m/sec.
V_d = the aerosol median droplet volume in mL.

C.12. AEROSOL CHAMBER BACKGROUND PARTICLE CONSIDERATIONS.

a. While conducting testing in bio-aerosol chambers, it is important to keep the concentration of chamber background particles as low as possible to avoid affecting the performance of the biological point detector being tested. In addition, the presence of

background particles during challenge aerosol dissemination skews the NMAD, MMAD, and aerosol concentration calculations. Chamber background particles are sometimes generated by electric motors belonging to the SUT or to referee instrumentation electric motors, or the particles may be residual from previous testing in the chamber.

b. If the average particle distributions of the challenge aerosol and background particles are known, then the background particles included in the data collected by the APS™ can be subtracted from the NMAD, MMAD, and aerosol concentration calculations. In general, the particle distribution of the challenge aerosol without background particles is dependent on the nozzle used to aerosolize the slurry, the type of slurry, and the target size of the particle being aerosolized.

(1) A background particle distribution should be determined before testing begins. Background samples are collected as long as the test officer ensures that the background concentration appears stable for at least 2 minutes. Once the concentration is stable for 2 minutes, background samples are collected for 3 minutes.

(2) The background distribution is generated by averaging the number of particles in each size bin collected during the 3-minute background data collection period (Equation C-24).

$$B_i = \frac{1}{N}\sum_{\tau=1}^{\tau_{end}} b_{\tau,i} \qquad \textit{(Equation C-24)}$$

where:

B_i = the average background particle count for the i^{th} bin.
N = the number of particle size bins.
$b_{\tau,i}$ = the number of particles in each size bin at each time step.
τ = the index of each time step.
τ_{end} = the index of the last time step (it is also the total number of time steps).

(3) The data collected during a trial is corrected by subtracting the background distribution (Equation C-24) by using Equation C-25. This process is visually depicted in Figure C-2.

$$a_{\tau,i} = b_{\tau,i} - B_i;\ 1 \le i \le N \qquad \textit{(Equation C-25)}$$

where:

$a_{\tau,i}$ = the background corrected to the ith particle size at time t.
$b_{\tau,i}$ = the number of particles in each size bin at each time step.
B_i = the average background particle count for the ith bin.
N = the number of particle size bins.
i = the index of each particle size bin.

(4) An estimate of the NMAD, MMAD, and aerosol concentrations can then be obtained from this synthetic distribution.

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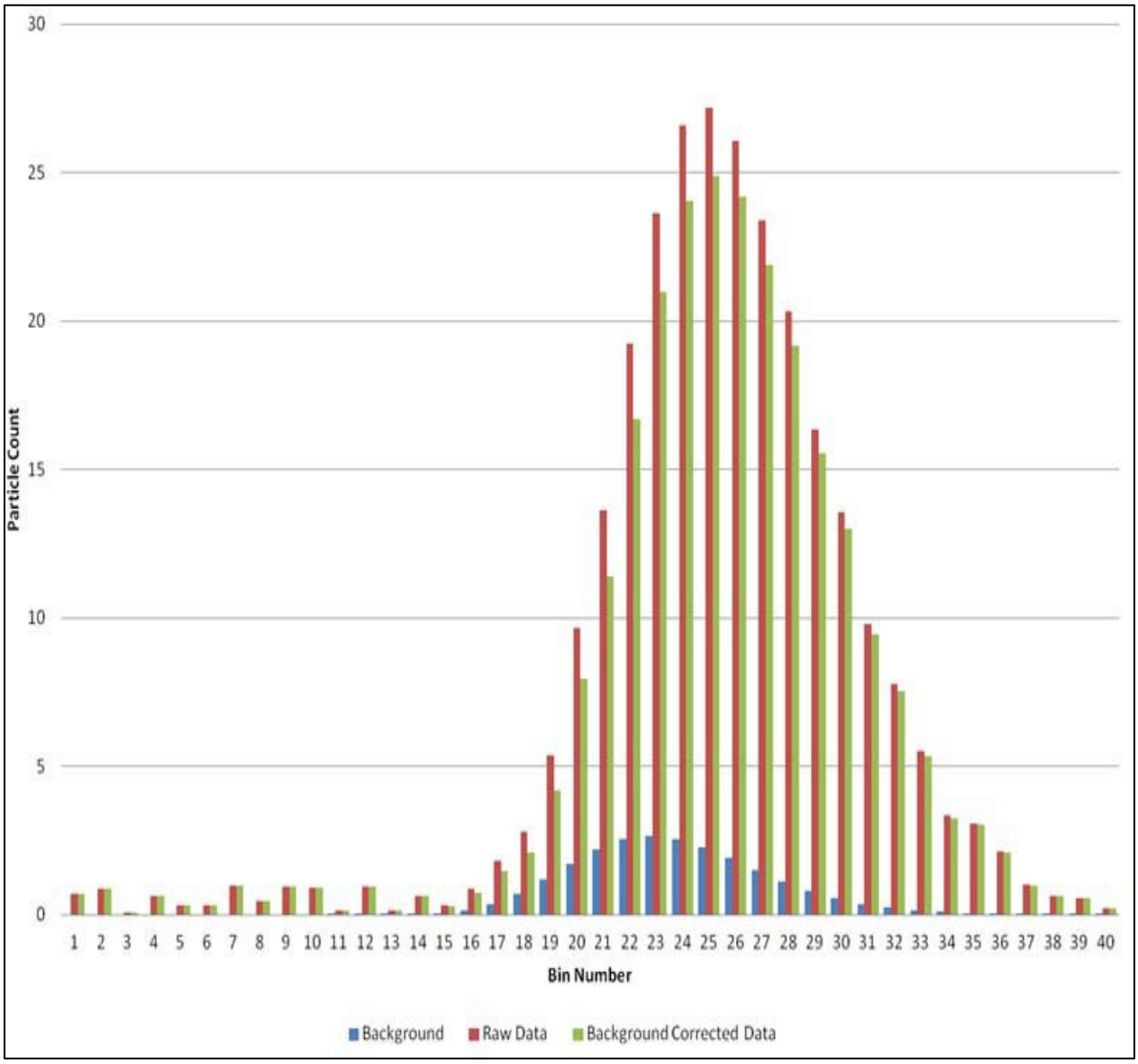

Figure C-2. Process of subtracting the background particle distribution from collected trial data.

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APPENDIX D. WIBS DATA HANDLING.

FIGURE LIST

APPENDIX D. WIBS DATA HANDLING.

D.1. UNITS OF MEASURE.

FPF provides a measure if the number of fluorescent aerosol particles relative to the total particle concentration. Percent fluorescence (%F) provides the same metric but not as a fraction (%F = FPF × 100). The metrics provide a quantitative measure of the amount of primary biological aerosol particles in a test sample. The FPF can be calculated for all particle sizes, or for a portion of the particle size range. Literature publications point to "coarse biological particles" as being the most relevant, those > 1.0 μm.

D.2. WIBS DATA REDUCTION AND CALCULATIONS.

a. Native raw data from the WIBS is recorded to a HDF5 data file. This is what is termed a Level 0 data product. The WIBS records data on a single-particle basis, meaning each record in the data file contains information for one particle that passed through the sample inlet and was counted using a continuous-wave 635 nm diode laser. When the diode laser detects a particle, it makes a measurement using a quadrant PMT. Each quadrant measures an intensity level, which is recorded and those signals are used by the WIBS to compute the particle size. When a particle is measured by the diode laser, it triggers the two Xenon flash lamps (Xe1 and Xe2). The Xenon flash lamps operate at 125 Hz, which means they can be triggered every 40 msec. At particle concentrations over about 5,000 ppL of air, particles enter the WIBS at a faster rate than the flash lamps can be triggered. This means that there are two particle counts that need to be tracked: 1) the number particles counted using the diode laser, N_P, and 2) the number of particles excited by the flash lamps, N_E. The total particle count, N_P, is used to compute the overall particle concentration. The "excited" particle count, N_E, is used as the denominator to compute the FPF. Note that "excited" does not mean the particle emitted a fluorescent signal. It simply means that the particle was stimulated by the Xenon flash lamp. The N_P and N_E counts will always be different at concentrations > ~5,000 ppL, with $N_E < N_P$. The vendor reports a 10 percent coincidence for N_P at 9.5×10^6 ppL, and 10 percent coincidence for N_E at 0.47×10^6 ppL, which are levels well above the particle concentration range used in testing.

b. The raw WIBS HDF5 file contains single-particle data formatted as shown in Figure D-1. Each field has a comment to the right that describes its content paraphrased from the WIBS Operator Manual[1]. The critical fields are *Seconds* (timestamp), *Size_um* (particle size), *XE1_FluorPeak* and *XE2_FluorPeak* (particle fluorescence data), and *Flag_Excited* (flag that indicates if particle was excited by the flash lamps).

APPENDIX D. WIBS DATA HANDLING.

```
Group '/'
    Group '/NEO'
        Group '/NEO/ParticleData'
            Dataset 'Asphericity'        % shape factor from quadrant detector
            Dataset 'Density_g_cm3'      % user-adjustable constant for sizing (1 g/cm3 default)
            Dataset 'EP_Overflow_Flag'   % excited particle overflow flag - not used
            Dataset 'Flag_Excited'       % =1 if particle was excited by flashlamp, else =0
            Dataset 'Mass_ug'            % particle mass computed from size and density
            Dataset 'NF_Shape_0'         % ADC value in quadrant 1 of 4-channel PMT
            Dataset 'NF_Shape_1'         % ADC value in quadrant 2 of 4-channel PMT
            Dataset 'NF_Shape_2'         % ADC value in quadrant 3 of 4-channel PMT
            Dataset 'NF_Shape_3'         % ADC value in quadrant 4 of 4-channel PMT
            Dataset 'NF_Sizer_Relative_Peak'       % NF ADC value above NF baseline
            Dataset 'NF_Sizer_Transit_Time_nsec'   % Transit time of particle through laser
            Dataset 'Seconds'            % particle timestamp
            Dataset 'Size_um'            % calculated particle size
            Dataset 'Xe1_FluorPeak'      % fluorescence intensity from Xe1 excitation (2D)
            Dataset 'Xe2_FluorPeak'      % fluorescence intensity from Xe2 excitation (2D)
```

Figure D-1. WIBS HDF5 raw (Level 0) particle data.

NOTE: ADC – analog to digital converter; HDF5 – Hierarchical Data Format version 5; NF – fluorescent particle counts; PMT – photomultiplier tube; 2D – 2 dimensional.

c. There is also another data group in the HDF5 file called *'/NEO/MonitoringData'* that contains instrument monitoring data such as circuit board temperatures, flash lamp power, and mass flow rates. These are helpful to diagnose instrument problems but are not relevant to processing of the particle data, so are not described here. Refer to the WIBS® Operator Manual[1] for details.

d. During operation, the WIBS can be programmed to record what is termed a Forced Trigger (FT) file, which provides a data set that contains the baseline PMT noise for each of the three fluorescence data channels. This is important to the Level 0 data processing because particle fluorescence is determined relative to the PMT noise. Typically a particle is declared to be fluorescent if the PMT peak signal (i.e., Xe1_FluorPeak in the data file) is greater than 3 times the standard deviation ($> 3\sigma$) of the PMT noise. The data collected during a test trial will include one or more FT reference files, and one or more data files depending on how the WIBS is programmed by the user to record data.

e. First, the FT file is processed. Data in the two-dimensional fields *Xe1_FluorPeak* and *Xe2_FluorPeak* are read into three arrays corresponding to fluorescence channels A, B, and C: *x1c1 = Xe1_FluorPeak(1, n)*, *x1c2 = Xe1_FluorPeak(2, n),* and *x2c2 = Xe2_FluorPeak(2, n)*. Where *n* is the number of samples in the FT file. Outliers are removed and the mean and standard deviation are computed for each channel (μ_i and σ_i where i is the channel subscript). These computed values will be used as inputs to the trial data processing which comes next.

f. To process the trial file(s) the fluorescence data in the *Xe1_FluorPeak* and *Xe2_FluorPeak* fields are read into three arrays as described above. In addition to that, three

other fields are read: *Size_um*, *Flag_Excited*, and *Seconds*. These are all one-dimensional arrays of size, N_P, the number of particles sampled by the WIBS. First, the particle size data in *Size_um* is sorted into 52 size bins from 0.5 - 20.0 µm. The size bin table is shown in Figure D-2. The TSI, Inc. APS™ table is used for convenience so WIBS data can be easily compared to referee data from the APS™.

APS Size Bins			
Bin	Lower Bin Edge	Bin Center	Upper Bin Edge
1	0.486	0.523	0.523
2	0.523	0.542	0.562
3	0.562	0.583	0.604
4	0.604	0.626	0.649
5	0.649	0.673	0.698
6	0.698	0.724	0.750
7	0.750	0.777	0.806
8	0.806	0.836	0.866
9	0.866	0.898	0.931
10	0.931	0.965	1.000
11	1.000	1.037	1.075
12	1.075	1.114	1.155
13	1.155	1.197	1.241
14	1.241	1.287	1.334
15	1.334	1.383	1.433
16	1.433	1.486	1.540
17	1.540	1.597	1.655
18	1.655	1.715	1.778
19	1.778	1.843	1.911
20	1.911	1.981	2.054
21	2.054	2.129	2.207
22	2.207	2.288	2.371
23	2.371	2.458	2.548
24	2.548	2.641	2.738
25	2.738	2.839	2.943
26	2.943	3.051	3.162

APS Size Bins			
Bin	Lower Bin Edge	Bin Center	Upper Bin Edge
27	3.162	3.278	3.398
28	3.398	3.523	3.652
29	3.652	3.786	3.924
30	3.924	4.068	4.217
31	4.217	4.372	4.532
32	4.532	4.698	4.870
33	4.870	5.048	5.233
34	5.233	5.425	5.623
35	5.623	5.829	6.043
36	6.043	6.264	6.494
37	6.494	6.732	6.978
38	6.978	7.234	7.499
39	7.499	7.774	8.058
40	8.058	8.354	8.660
41	8.660	8.977	9.306
42	9.306	9.647	10.000
43	10.000	10.366	10.746
44	10.746	11.140	11.548
45	11.548	11.971	12.409
46	12.409	12.864	13.335
47	13.335	13.824	14.330
48	14.330	14.855	15.399
49	15.399	15.963	16.548
50	16.548	17.154	17.783
51	17.783	18.435	19.110
52	19.110	19.810	20.535

Figure D-2. Aerosol Particle Sizer (APS™) size bin table.

g. Before fluorescence data can be processed, the subset of particles excited by the flash lamps needs to be extracted from the *Size_um* array using the *Flag_Excited* data. The indices of the *Size_um* array that contain excited particles are the same indices of the *Flag_Excited* array that contain the value of 1. The subset will be an array with N_E particles. There will be three new arrays, one for each fluorescence channel A, B, and C. The next step is to go through the three arrays and sort into the 52 size bins.

h. The process described above is conducted using a sample interval. The *Seconds* field is used to group the particles into the interval. Typically a 10-second interval is used to provide good temporal fidelity.

i. The overall process is summarized in Figure D-3. The output is classified as Level 2 product because it does not have physical units associated with the data such as concentration (ppL) or fluorescence percentage. It only contains particle counts and timestamps.

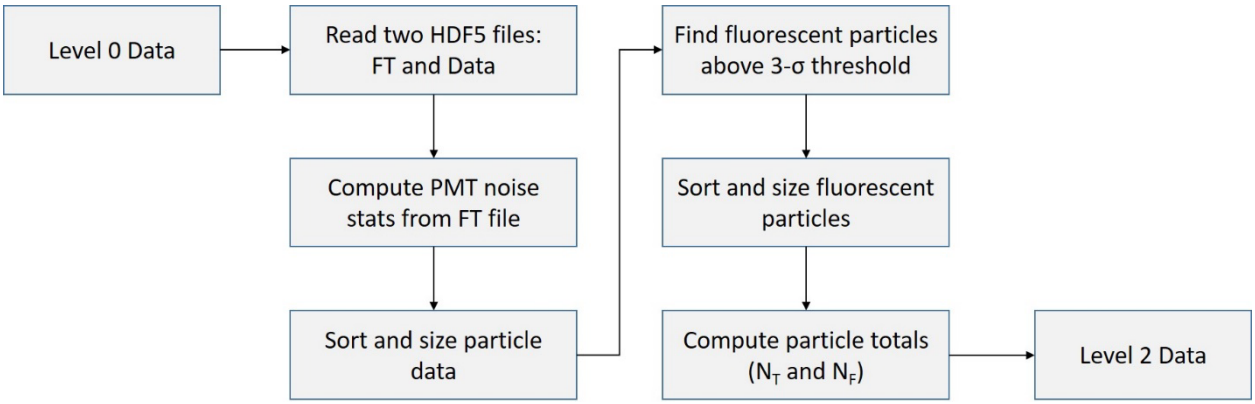

Figure D-3. Wideband Integrated Bioaerosol Sensor (WIBS) data processing flow chart.

NOTE: HDF5 - Hierarchical Data Format version 5; PMT - photomultiplier tube.

j. An example Level 2 WIBS data product is shown in Figure D-4. This tab-delimited ASCII text format can be analyzed and visualized by Excel or any number of other commercially available data processing tools such as Matlab, R, or Python.

k. Each row in the file represents one WIBS sample, processed at a user-specified time interval (typically 10 seconds). The columns contain the particle counts for each processed sample. Columns 1 and 2 contain the sample timestamp. Columns 3 and 4 contain the total and excited particle counts (N_P and N_E, see supporting math in next section). Columns 5 through 7 contain the fluorescent particle counts, N_F, for Channels A, B, and C respectively. The next series of columns contain five PSD that characterize the WIBS data. Columns 8 through 59 contain the particle counts within each size bin and thus describes the total particle size distribution (TPSD) for N_P. Columns 60 through 111 contain the excited particle size distribution (EPSD) for N_E. Columns 112 through 163, 164 through 215, and 216 through 268 contain the

APPENDIX D. WIBS DATA HANDLING.

FPSD for channels A, B, and C respectively. Row 1 contains the column headers, with the particle size bin centers as header elements for each of the 52 bins for the 5 PSDs.

							52 Bins TPSD				52 Bins EPSD				52 Bins FPSD A				52 Bins FPSD B				52 Bins FPSD C			
Date	Time	Total Counts	Excited Counts	Ch A Counts	Ch B Counts	Ch C Counts	0.523	0.542	...	19.81	0.523	0.542	...	19.81	0.523	0.542	...	19.81	0.523	0.542	...	19.81	0.523	0.542	...	19.81
31/03/2021	17:19:23	2	2	0	0	0	2	0	...	0	2	0	...	0	0	0	...	0	1	0	...	0	1	0	...	0
31/03/2021	17:19:34	2	2	0	1	0	1	0	...	0	1	0	...	0	0	0	...	0	1	0	...	0	1	0	...	0
31/03/2021	17:19:44	7	7	1	0	0	3	2	...	0	3	2	...	0	1	1	...	0	1	1	...	0	0	0	...	0
31/03/2021	17:19:55	4	4	0	0	0	5	1	...	0	5	1	...	0	0	0	...	0	0	0	...	0	0	0	...	0
31/03/2021	17:20:05	1	1	0	0	0	2	0	...	0	2	0	...	0	0	0	...	0	1	0	...	0	1	0	...	0
31/03/2021	17:20:16	4	4	1	3	2	1	0	...	0	1	0	...	0	0	0	...	0	1	0	...	0	1	0	...	0
31/03/2021	17:20:27	6	6	0	2	1	3	2	...	0	3	2	...	0	0	0	...	0	1	0	...	0	0	0	...	0

Figure D-4. Example Level 2 WIBS data file containing particle counts and size distributions.

NOTE: EPSD - excited particle size distribution; FPSD - fluorescent particle size distribution; TPSD - total particle size distribution.

D.3. SUPPORTING MATH.

a. The supporting math for the computations involved in generating the Level 2 product are shown below in Equations D-1 through D-4. Computations to generate Level 3 metrics of concentration and fluorescence percentage are shown in equations D-5 through D-7.

b. The total number of particles in one sample is computed using Equation D-1. This is the number of particles detected by the diode laser at the inlet, which is a function of particle size. The total number of samples is computed as the sum across particle size bins 2 through 52 to remain consistent with the APS™ data, in which size bin number 1 is ignored.

$$N_T = \sum_{z=2}^{52} n_P(z) \qquad \textit{(Equation D-1)}$$

where:

N_T = total number of particles
z = size bin index (which ranges from 2 through 52, see Figure D-4)
n_P = total particle size distribution (TPSD)

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c. During processing of Level 0 raw WIBS data, Equation D-2 is used to determine if a particle was fluorescent or not, when it was excited by the flash lamps. A threshold must be set to make this determination. That threshold is computed for each WIBS fluorescence channel: A, B, and C. It is based on the mean (μ_{FT}) and standard deviation (σ_{FT}) of the PMT noise. The fluorescence threshold is typically set for $3\sigma_{FT}$. Equation D-2 operates on a sequence of particles that were collected during a user-specified time interval (typically 10 seconds). That sequence is determined by examining the particles that were excited by the flash lamps during the interval.

$$n_F(z) = \left(v(i,z) > (\mu_{FT} + 3\sigma_{FT})\right) \ , \ \ i = 1, \ldots, K \qquad \textit{(Equation D-2)}$$

where:

n_F = FPSD
i = index of individual particle record in raw WIBS file
z = particle size
v = PMT voltage level of individual particle excited by the flash lamps
μ_{FT} = mean PMT noise
σ_{FT} = standard deviation of PMT noise
K = number of particles excited by the flash lamps

NOTE: The values in $n_F(z)$ are the resulting FPSDs for WIBS channels A, B, and C in the Level 2 ASCII in Figure D-4.

d. The total number of fluorescent particles (N_F) in one sample is computed using Equation D-3, which operates on the results of Equation D-2 by adding up the particle counts in the FPSD for each channel, A, B, or C. The sum is computed using a large particle index for the bin associated with 0.8 µm or 1.0 µm (i.e., 8 or 11 from the table in Figure D-4).

$$N_F = \sum_{z=c}^{52} n_F(z) \qquad \textit{(Equation D-3)}$$

where:

N_F = total number of fluorescent particles
z = size bin index (which ranges from c through 52, see Figure D-4)
n_F = fluorescence particle size distribution (FPSD A, B, or C)
c = large particle cutoff index bin (typically 0.8 or 1.0 µ) from table in Figure D-4.

APPENDIX D. WIBS DATA HANDLING.

e. The number of particles excited by the flash lamps in one sample, N_E, are calculated over the full particle range. Size bin number 1 is ignored by convention to maintain compatibility with the APS™.

$$N_E = \sum_{z=2}^{52} n_E(z) \qquad \textit{(Equation D-4)}$$

where:

N_E = total number of fluorescent particles
z = size bin index (which ranges from 2 through 52, see Figure D-4)
n_E = excited particle size distribution (EPSD)

NOTE: The number of particles excited by the flash lamps, N_E, is not the same as those counted by the diode laser in Equation D-1. This is an important distinction that is critical to take into account when computing the fluorescent particle fraction in Equation D-5.

$$FPF = {}^{N_F}/_{N_E} \qquad \textit{(Equation D-5)}$$

where:

FPF = the fluorescent particle fraction
N_F = total number of fluorescent particles
N_E = total number particles excited by the flash lamps

f. The FPF is computed as the number of fluorescent particles, N_F, divided by the total number of particles excited by the flash lamps, N_E. **NOTE**: it is critical to use N_E in the denominator this calculation as it can be very different from the total number of particles, N_P, sampled by the WIBS®.

g. Often the FPF is expressed as a percentage of fluorescent particles. The percent fluorescence is computed in Equation D-6.

$$\%F = FPF \times 100 \qquad \textit{(Equation D-6)}$$

where:

$\%F$ = percent fluorescence
FPF = the fluorescent particle fraction

APPENDIX D. WIBS DATA HANDLING.

h. Particle concentration is computed (Equation D-7) based on the sample flow rate of 0.3 L/min and the sample interval at which the Level 0 data are processed.

$$c_i = \frac{60}{Q\,\tau_s} \times n_i \qquad \textit{(Equation D-7)}$$

where:

c_i = is particle concentration (ppL) for individual sample, i
Q = sample flow rate of the WIBS (0.3 L/min)
τ_s = data sample interval in seconds (typically 10 seconds)
n_i = particle count for individual sample i

D.4. WIBS REFEREE REPORT.

a. A standard data report can be generated for each chamber test trial. The live agent test method introduces a synthetic natural background to the chamber to simulate the operational environment. After the synthetic background stabilizes, a prescribed amount of live agent is disseminated. The level of background and agent is sustained for a prescribed time period, then the background dissemination is halted. The three periods are 1) background only, 2) background + agent, and 3) agent only. The WIBS is used to assess particle fluorescence during each period, and it also can monitor particle concentration. It can also measure particle size distribution and fluorescent particle size distribution. The summary report plots are shown in Figure D-5.

APPENDIX D. WIBS DATA HANDLING.

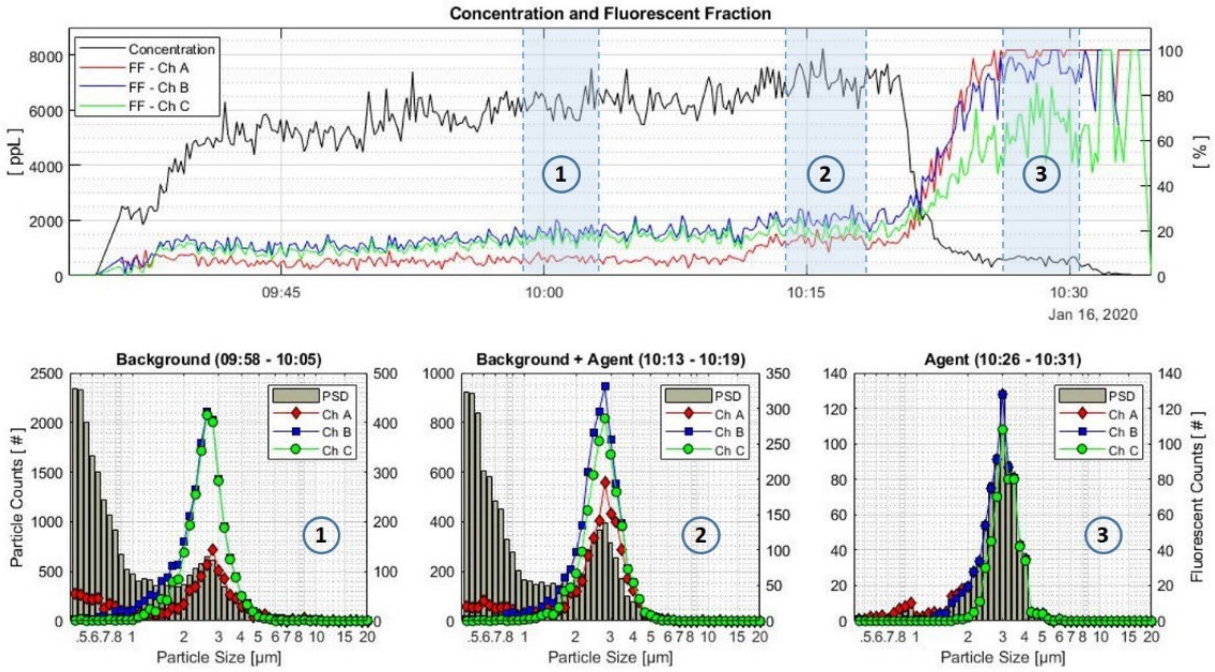

Figure D-5. WIBS Chamber Test Report[3] Sample Data.

NOTE: Concentration vs. Fluorescence Percentage (top) and Particle Size Distribution Plots (Bottom) for Three regions during the test: 1) background, 2) background + agent, and 3) agent only.

b. The concentration and percent fluorescence versus time plot can be generated from the Level 2 product in Figure D-2 using Equations D-5 through D-7.

c. Particle concentration is computed by applying Equation D-7 to the Total Counts figure in Column 3. For this example the sample flow rate is 0.3 L/min and sample interval is 10 seconds, so it is a simple matter of multiplying the Total Counts by 20.

d. Particle fluorescence percentage is computed using Equations D-5 and D-6. First the channel fluorescent particle counts in columns 5, 6, and 7 (Figure D-4) are each divided by the Excited Counts in column 4 (Equation D-5) to compute the FPF. The result is then multiplied by 100 (Equation D-6) to get the FPF into units of percent (%).

APPENDIX E. REFERENCES.

1. Droplet Measurement Technologies Inc., Longmont, Colorado, *Wideband Integrated Bioaerosol Sensor New Electronics Option (WIBS-NEO) Operator Manual*, DOC-0417 Rev D, 8 April 2020.

2. Droplet Measurement Technologies Inc., Longmont, Colorado, DOC-0433,*WIBS-NEO Toolkit*, Revision B.

3. D'Amico, F. M., Sutton, T. S., Deluca, P., Hurley, J. M., Ferguson, K., *Wideband Integrated Bioaerosol Sensor (WIBS) Verification and Validation (V&V) for Chamber Testing – Final Report*, CCDC CBC-TR-1680, May 2020.

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APPENDIX F. APPROVAL AUTHORITY.

CSTE-CI (73-1jj) 29 September 2022

MEMORANDUM FOR

Commander, U.S. Army White Sands Missile Range
Executive Director, U.S. Army Evaluation Center
Commander, U.S. Army Operational Test Command
Commander, U.S. Army Yuma Proving Ground
Commander, U.S. Army Dugway Proving Ground
Commanders, U.S. ATEC Test Centers
Director, U.S. ATEC Tropic Regions Test Center
Director, U.S. ATEC West Desert Test Center

SUBJECT: Test Operations Procedure 08-2-066B Aerosol Testing of Biological Point Detection Systems

1. Test Operations Procedure (TOP) 08-2-066B Aerosol Testing of Biological Point Detection Systems, 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 provides guidelines for designing and conducting indoor and outdoor chamber tests involving the aerosolization of biological warfare agents (BWAs) and simulants, and for collecting referee data using currently available referee equipment. The test procedures in this TOP define a methodology to test the performance of biological point detection systems and their components using representatives from each of the four classes of BWAs/biological materials (bacterial spores, vegetative bacteria, viruses, and toxins).

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 Digitally signed by ZWIEBEL.MICHAEL.J.1229197289 Date: 2022.10.04 11:36:30 -04'00'

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

APPENDIX F. APPROVAL AUTHORITY.

TECMIPT Test Operations Procedure (TTOP)

08-2-066B Aerosol Testing of Biological Point Detectors

Biological Capability Area Process Action Team (CAPAT):

CAPAT Review & Concurrence: September 2021

Test and Evaluation Capabilities and Methodologies Integrated Process Team (TECMIPT) Participants:

DISTRIBUTION A. Approved for public release: distribution unlimited.

REFERENCES:
(a) *Chemical and Biological Defense Program (CBDP) Test and Evaluation (T&E) Standards Development Plan*, dated 19 July 2010.
(b) *Memorandum of Understanding (MOU) Among the Department of National Defence of Canada the Secretary of State for Defense of the United Kingdom of Great Britain and Northern Ireland and the Secretary of Defense on Behalf of the Department of Defense of the United State of America concerning the Research, Development and Acquisition of Chemical, Biological and Radiological Defense Materiel*, dated June 2000. Amendment One, dated August 2006.

APPENDIX F. APPROVAL AUTHORITY.

Sep 2021

TECMIPT Test Operations Procedure (TTOP)
08-2-066B Aerosol Testing of Biological Point Detectors

The Biological Capability Area Process Action Team (CAPAT) recommends approval of the TECMIPT Test Operations Procedure (TTOP) 08-2-066B Aerosol Testing of Biological Point Detectors. 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)	FRIESE.AMY.JEAN.1127559216 Digitally signed by FRIESE.AMY.JEAN.1127559216 Date: 2022.09.27 12:29:28 -04'00' Amy Friese	
Joint Program Executive Office of Chemical Biological Defense (JPEO-CBD) Test & Evaluation	RYBAK.JOSEPH.1364953735 Digitally signed by RYBAK.JOSEPH.1364953735 Date: 2022.06.09 10:55:17 -04'00' Joseph Rybak	
Joint Requirements Office for Chemical, Biological, Radiological and Nuclear Defense (JRO-CBRND)	BULSON.CHRISTOPHER.D.1236325462 Digitally signed by BULSON.CHRISTOPHER.D.1236325462 Date: 2022.05.16 09:38:22 -04'00' Lt. Col Christopher D. Bulson	16 May 2022
Joint Science and Technology Office (JSTO)	ODELL.BRETT.KYLE.1274940553 Digitally signed by ODELL.BRETT.KYLE.1274940553 Date: 2021.09.29 15:07:38 -04'00' Brett Odell	29 Sept 21
US Army Evaluation Center (AEC)	HUGHES.JULIANE.OLSEN.1285055837 Digitally signed by HUGHES.JULIANE.OLSEN.1285055837 Date: 2021.02.17 12:47:55 -05'00' Juliane Hughes	
Operational Test and Evaluation Force (OPTEVFOR)	Joseph Thiering	9FEB2022
Air Force Operational Test and Evaluation Center (AFOTEC)	Megan R DeLeon MSgt Megan R DeLeon	19 Oct 21
Marine Corps Operational Test & Evaluation Activity (MCOTEA)	WADLEY.MICHAEL.CRAIG.1130810841 Digitally signed by WADLEY.MICHAEL.CRAIG.1130810841 Date: 2022.01.18 19:15:05 -05'00' Michael Wadley	
Bio CAPAT Co-Chair	NIBLICK.CHRISTOPHER.JAMES.1256873113 Digitally signed by NIBLICK.CHRISTOPHER.JAMES.1256873113 Date: 2022.02.23 15:28:51 -05'00' Chris Niblick	
Bio CAPAT Co-Chair	HARPER.STEPHEN.EARL.1042133112 Digitally signed by HARPER.STEPHEN.EARL.1042133112 Date: 2022.01.18 16:28:32 -05'00' Stephen Harper	
Bio CAPAT Co-Chair	HURLEY.JOHN.MICHAEL.1291223652 Digitally signed by HURLEY.JOHN.MICHAEL.1291223652 Date: 2022.04.19 14:44:03 -06'00' Dr. Michael Hurley	

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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, US Army Dugway Proving Ground (TEDP-TD-QM), Dugway, Utah 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, Virginia 22060-6218. This document is identified by the accession number (AD No.) printed on the first page.