AOAC SMPR® 2016.006

Standard Method Performance Requirements (SMPRs) for DNA-Based Methods of Detecting *Bacillus anthracis* **in Field-Deployable, Department of Defense Aerosol Collection Devices**

Intended Use: Field-deployed use for analysis of aerosol collection filters and/or liquids

1 Applicability

Detection of *Bacillus anthracis* in collection buffers from aerosol collection devices. Field-deployable assays are preferred.

2 Analytical Technique

Molecular detection of nucleic acid.

3 Definitions

Acceptable minimum detection level (AMDL).—The predetermined minimum level of an analyte, as specified by an expert committee that must be detected by the candidate method at a specified probability of detection (POD).

Environmental factors.—For the purposes of this SMPR: Any factor in the operating environment of an analytical method, whether abiotic or biotic, that might influence the results of the method.

Exclusivity.—Study involving pure non-target strains, which are potentially cross-reactive, that shall not be detected or enumerated by the candidate method.

Inclusivity.—Study involving pure target strains that shall be detected or enumerated by the candidate method.

Interferents.—A . . . substance in analytical procedures . . . that, at a (the) given concentration, causes a systematic error in the analytical result (International Union of Pure and Applied Chemistry Analytical Chemistry Division Commission on Analytical Reactions and Reagents Definition and Classification of Interferences in Analytical Procedures Prepared for Publication by W.E. Van Der Linden, *Pure Appl. Chem*. **61**(1), 91–95(1989). Printed in Great Britain, 1989, IUPAC). Sometimes also known as interferants.

Maximum time-to-result.—Maximum time to complete an analysis starting from the collection buffer to assay result.

Probability of detection (POD).—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a specified analyte level or concentration with a ≥0.95 confidence interval.

System false-negative rate.—Proportion of test results that are negative contained within a population of known positives.

System false-positive rate.—Proportion of test results that are positive contained within a population of known negatives.

4 Method Performance Requirements

See Table 1.

Table 1. Method performance requirements

Parameter	Minimum performance requirement
AMDL	2000 standardized BA Ames spores per mL liquid in the candidate method sample collection buffer
Probability of detection at AMDL within sample collection buffer	≥0.95
Probability of detection at AMDL in environmental matrix materials	≥0.95
System false-negative rate using spiked environmental matrix materials	≤5%
System false-positive rate using environmental matrix materials	≤5%
Inclusivity	All inclusivity strains (Table 3) must test positive at 2x the AMDL[a]
Exclusivity	All exclusivity strains (Table 4 and Annex 1—Part 2) must test negative at 10x the AMDL[a]

[a] 100% correct analyses are expected. All discrepancies are to be retested following the *AOAC Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* [*Official Methods of Analysis of AOAC INTERNATIONAL* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, USA, Appendix I, http://www.eoma.aoac.org/app_i.pdf].

Table 2. Controls

Control	Description	Implementation
Positive	Designed to demonstrate an appropriate test response. The positive control should be included at a low but easily detectable concentration, and should monitor the performance of the entire assay. The purpose of using a low concentration of positive control is to demonstrate that the assay sensitivity is performing at a previously determined level of sensitivity.	Single use per sample (or sample set) run
Negative	Designed to demonstrate that the assay itself does not produce a detection in the absence of the target organism. The purpose of this control is to rule out causes of false positives, such as contamination in the assay or test.	Single use per sample (or sample set) run
Inhibition	Designed to specifically address the impact of a sample or sample matrix on the assay's ability to detect the target organism.	Single use per sample (or sample set) run

Table 3.	Inclusivity panel				
No.	Cluster	Genotype	Strain	Origin	Characteristics
1	A1a	7	Canadian bison	Wood bison	pXO1$^+$, pXO2$^+$, VNTR[a] genotype group A1a
2	A3a	45[b]	V770-NP-1R	Vaccine (United States)	pXO1$^+$, pXO2$^-$, VNTR genotype group A3A
3	A2	29	PAK-1	Sheep (Pakistan)	pXO1$^+$, pXO2$^+$, VNTR genotype group A2
4	A3a	51	BA1015	Bovine (MD)	pXO1$^+$, pXO2$^+$, VNTR genotype group A3a
5	A3b	62	Ames	Bovine (Texas)	pXO1$^+$, pXO2$^+$, VNTR genotype group A3b
6	A3c	67	K3	South Africa	pXO1$^+$, pXO2$^+$, VNTR genotype group A3c
7	A3d	68	Ohio ACB	Pig	pXO1$^+$, pXO2$^+$, VNTR genotype group A3d
8	A4	69	SK-102 (Pakistan)	Imported wool	pXO1$^+$, pXO2$^+$, VNTR genotype group A4
9	A4	77	Vollum 1B	USAMRIID[c]	pXO1$^+$, pXO2$^+$, VNTR genotype group A4
10	B1	82	BA1035	Human (South Africa)	pXO1$^+$, pXO2$^+$, VNTR genotype group B1
11	B2	80	RA3	Bovine (France)	pXO1$^+$, pXO2$^+$, VNTR genotype group B2
12	A1a	8	Pasteur	USAMRIID	pXO1$^-$, pXO2$^+$, VNTR genotype group A1a
13	A3b	59, 61[b]	Sterne	USAMRIID	pXO1$^+$, pXO2$^-$, VNTR genotype group A3b
14	A1b	23	Turkey No. 32	Human (Turkey)	pXO1$^+$, pXO2$^+$, VNTR genotype group A1b

[a] VNTR = Variable number tandem repeat.

[b] Organism contains only seven of eight multiple locus variable number tandem repeat analysis (MLVA) markers due to the absence of pXO2. Genotypes listed are consistent with seven of the eight markers.

[c] USAMRIID = United States Army Medical Research Institute for Infectious Diseases.

5 System Suitability Tests and/or Analytical Quality Control

The controls listed in Table 2 shall be embedded in assays as appropriate. Manufacturer must provide written justification if controls are not embedded in the assay.

6 Validation Guidance

Official Methods of Analysis (2016) Appendix I: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures*, AOAC INTERNATIONAL, Rockville, MD, USA.

Inclusivity and exclusivity panel organisms used for evaluation must be characterized and documented to truly be the species and strains they are purported to be.

7 Maximum Time-to-Result

Within 4 h.

8 Guidance on Combining DNA for Exclusivity Evaluation

DNA from exclusivity panel organisms 1–9 in Table 4 may be tested as isolated DNA, or combined to form a pool of exclusivity panel organisms, with each panel organism represented at 10 times the AMDL. If an unexpected result occurs, each of the exclusivity organisms from a failed pool must be individually retested at 10 times the AMDL.

DNA from exclusivity panel organisms 10–15 in Table 4 *cannot* be combined for exclusivity evaluation.

Approved by the AOAC Stakeholder Panel on Agent Detection Assays (SPADA). Final Version Date: March 22, 2016.

Table 4.	Exclusivity panel (near-neighbor)		
No.	Species	Strain	Plasmid status
1	*B. cereus*	S2-8	pXO1$^-$, pXO2$^-$
2	*B. cereus*	3A	pXO1$^-$, pXO2$^-$
3	*B. thuringiensis*	HD1011	pXO1$^-$, pXO2$^-$
4	*B. thuringiensis*	HD682	pXO1$^-$, pXO2$^-$
5	*B. cereus*	D17	pXO1$^-$, pXO2$^-$
6	*B. thuringiensis*	HD571	pXO1$^-$, pXO2$^-$
7	*B. cereus*	Al Hakam	pXO1$^-$, pXO2$^-$
8	*B. cereus*	ATCC 4342	pXO1$^-$, pXO2$^-$
9	*B. cereus*	FM1	pXO1$^-$, pXO2$^-$
10	*B. cereus*	E33L	pXO1$^-$, pXO2$^-$
11	*B. thuringiensis*	97-27	pXO1$^-$, pXO2$^-$
12	*B. cereus*	G9241	pBCXO1^{+a}, pXO2$^-$
13	*B. cereus*	03BB102	pXO1$^+$, capA$^+$, capB$^+$, capC^{+b}
14	*B. cereus*	03BB108	pX01$^+$, capA$^+$, capB$^+$, capC^{+b}

[a] pBCXO1 is pX01-like, but not identical.

[b] capA, capB, and capC are contained within the *Bacillus anthracis* pXO2 plasmid; however, the capA, capB, and capC sequences are found in strains 03BB102 and 03BB108 in the absence of the pxO2 plasmid.

Annex 1. Environmental Factors for Validating Biological Threat Agent Detection Assays

[Adapted from the Environmental Factors Panel approved by SPADA on June 10, 2010.]

The Environmental Factors Studies supplement the biological threat agent near-neighbor exclusivity testing panel. There are three parts to Environmental Factors Studies: Part 1—Environmental matrix samples; Part 2—Environmental organisms study; and Part 3—Potential interferents applicable to Department of Defense applications (added in June 2015 for the Department of Defense project).

Part 1: Environmental Matrix Samples— Aerosol Environmental Matrices

Method developers shall obtain environmental matrix samples that are representative and consistent with the collection method that is anticipated to ultimately be used in the field. This includes considerations that may be encountered when the collection system is deployed operationally such as collection medium, duration of collection, diversity of geographical areas that will be sampled, climatic/environmental conditions that may be encountered and seasonal changes in the regions of deployment.

Justifications for the selected conditions that were used to generate the environmental matrix and limitations of the validation based on those criteria must be documented.

• Method developers shall test the environmental matrix samples for interference using samples inoculated with a target biological threat agent sufficient to achieve 95% probability of detection.

• Cross-reactivity testing will include sufficient samples and replicates to ensure each environmental condition is adequately represented.

Part 2: Environmental Panel Organisms

This list is comprised of identified organisms from the environment.

Inclusion of all environmental panel organisms is not a requirement if a method developer provides appropriate justification that the intended use of the assay permits the exclusion of specific panel organisms. Justification for exclusion of any environmental panel organism(s) must be documented and submitted.

Organisms and cell lines may be tested as isolated DNA, or as pools of isolated DNA. Isolated DNA may be combined into pools of up to 10 panel organisms, with each panel organism represented at 10 times the AMDL, where possible. The combined DNA pools are tested in the presence (at 2 times the AMDL) and absence of the target gene or gene fragment. If an unexpected result occurs, each of the individual environmental organisms from a failed pool must be individually retested at 10 times the AMDL with and without the target gene or gene fragment at 2 times the AMDL in the candidate method DNA elution buffer.

DNA in this list that already appear in the inclusivity or exclusivity panel do not need to be tested again as part of the environmental factors panel.

- **Potential bacterial biothreat agents**
  Bacillus anthracis Ames
  Yersinia pestis Colorado-92
  Francisella tularensis subsp. *tularensis* Schu-S4
  Burkholderia pseudomallei
  Burkholderia mallei
  Brucella melitensis

- **Cultivatable bacteria identified as being present in air, soil, or water**
  Acinetobacter lwoffii
  Agrobacterium tumefaciens
  Bacillus amyloliquefaciens
  Bacillus cohnii
  Bacillus psychrosaccharolyticus
  Bacillus benzoevorans
  Bacillus megaterium
  Bacillus horikoshii
  Bacillus macroides
  Bacteroides fragilis
  Burkholderia cepacia
  Burkholderia gladoli
  Burkholderia stabilis
  Burkholderia plantarii
  Chryseobacterium indologenes
  Clostridium sardiniense
  Clostridium perfringens
  Deinococcus radiodurans
  Delftia acidovorans
  Escherichia coli K12
  Fusobacterium nucleatum
  Lactobacillus plantarum
  Legionella pneumophilas
  Listeria monocytogenes
  Moraxella nonliquefaciens
  Mycobacterium smegmatis
  Neisseria lactamica
  Pseudomonas aeruginosa
  Rhodobacter sphaeroides
  Riemerella anatipestifer
  Shewanella oneidensis
  Staphylococcus aureus
  Stenotophomonas maltophilia
  Streptococcus pneumoniae
  Streptomyces coelicolor
  Synechocystis
  Vibrio cholerae

- **Microbial eukaryotes**
  Freshwater amoebae:
    Acanthamoeba castellanii
    Naegleria fowleri

  Fungi:
    Alternaria alternata
    Aspergillus fumagatis
    Aureobasidium pullulans
    Cladosporium cladosporioides
    Cladosporium sphaerospermum
    Epicoccum nigrum
    Eurotium amstelodami
    Mucor racemosus
    Paecilomyces variotii
    Penicillum chrysogenum
    Wallemia sebi

- **DNA from higher eukaryotes**
  Plant pollen (if pollen is unavailable, vegetative DNA is acceptable):
  - *Zea mays* (corn)
  - *Pinus* spp. (pine)
  - *Gossypium* spp. (cotton)

  Arthropods:
  - *Aedes aegypti* (ATCC/CCL-125(tm) mosquito cell line)
  - *Aedes albopictus* (Mosquito C6/36 cell line)
  - *Dermatophagoides pteronyssinus* (Dust mite, commercial source)
  - *Xenopsylla cheopis* Flea (Rocky Mountain Labs)
  - *Drosophilia* cell line
  - *Musca domestica* (housefly; ARS, USDA, Fargo, ND, USA)
  - Gypsy moth cell lines [LED652Y cell line (baculovirus); Invitrogen]
  - Cockroach (commercial source)
  - Tick (Amblyomma and Dermacentor tick species for *F. tularensis* detection assays) (added by SPADA on March 22, 2016)

  Vertebrates:
  - *Mus musculus* (ATCC/HB-123) mouse
  - *Rattus norvegicus* (ATCC/CRL-1896) rat
  - *Canis familiaris* (ATCC/CCL-183) dog
  - *Felis catus* (ATCC/CRL-8727) cat
  - *Homo sapiens* (HeLa cell line ATCC/CCL-2) human
  - *Gallus gallus domesticus* (chicken)
  - *Capra hircus* (goat) (added by SPADA on September 1, 2015)

- **Biological insecticides**
  Strains of *B. thuringiensis* present in commercially available insecticides have been extensively used in hoaxes and are likely to be harvested in air collectors. For these reasons, it should be used to assess the specificity of these threat assays.

  - *B. thuringiensis* subsp. *israelensis*
  - *B. thuringiensis* subsp. *kurstaki*
  - *B. thuringiensis* subsp. *morrisoni*
  - Serenade (Fungicide) *B. subtilis* (QST713)

  Viral agents have also been used for insect control. Two representative products are:

  - Gypcheck for gypsy moths (*Lymanteria dispar* nuclear polyhedrosis virus)
  - Cyd-X for coddling moths (Coddling moth granulosis virus)
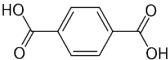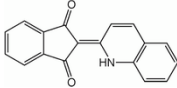
Part 3: Potential Interferents Study

The Potential Interferents Study supplements the Environmental Factors Study, and is applicable to all biological threat agent detection assays for Department of Defense applications. Table 5 provides a list of potential interferents that are likely to be encountered in various Department of Defense applications.

Method developers and evaluators shall determine the most appropriate potential interferents for their application. Interferents shall be spiked at a final test concentration of 1 µg/mL directly into the sample collection buffer. Sample collection buffers spiked with potential interferents shall be inoculated at 2 times the AMDL (or acceptable minimum identification level; AMIL) with one of the target biological threat agents.

Spiked/inoculated sample collection buffers shall be tested using the procedure specified by the candidate method. A candidate method that fails at the 1 µg/mL level may be reevaluated at lower concentrations until the inhibition level is determined.

It is expected that all samples are correctly identified as positive.

Table 5 is offered for guidance and there are no mandatory minimum requirements for the number of potential interferents to be tested.

Table 5. Potential interferents		
Compound		Potential theaters of operation
Group 1: Petroleum-based	JP-8[a]	Airfield
	JP-5[b]	Naval
	Diesel/gasoline mixture	Ground
	Fog oil (standard grade fuel number 2)	Naval, ground
	Burning rubber[c]	Ground, airfield
Group 2: Exhaust	Gasoline exhaust	Ground
	Jet exhaust	Naval, airfield
	Diesel exhaust	Ground
Group 3: Obscurants	Terephthalic acid[d]	Ground
	Zinc chloride smoke[e]	Ground
	Solvent yellow 33[f]	Ground
Group 4: Environmental	Burning vegetation	Ground, airfield
	Road dust	Ground
	Sea water (sea spray)	Naval
Group 5: Chemicals	Brake fluid[g]	All
	Brake dust[h]	Ground
	Cleaning solvent, MIL-L-63460[i]	All
	Explosive residues: High explosives[j], artillery propellant[k]	All

[a] JP-8: Air Force formulation jet fuel.

[b] JP-5: A yellow kerosene-based jet fuel with a lower flash point developed for use in aircraft stationed aboard aircraft carriers, where the risk from fire is particularly great. JP-5 is a complex mixture of hydrocarbons, containing alkanes, naphthenes, and aromatic hydrocarbons.

[c] Burning rubber (tire smoke): Gaseous C1-C5 hydrocarbons: methane; ethane; isopropene; butadiene; propane. Polycyclic aromatic hydrocarbons (58–6800 ng/m^3): parabenzo(a)pyrene; polychlorinated dibenzo-p-dioxins (PCDD); polychlorinated dibenzofurans (PCDF). Metals (0.7–8 mg/m^3): zinc; lead; cadmium.

[d] Terephthalic acid: Used in the AN/M83 hand grenade currently used by U.S. military.

[e] Zinc chloride smoke: Also known as "zinc chloride smoke" and "HC smoke." Was used in the M8 grenade and still used in 155 mm artillery shells. HC smoke is composed of 45% hexachloroethane, 45% zinc oxide, and 10% aluminum.

[f] Solvent yellow 33 [IUPAC name: 2-(2-quinolyl)-1,3-indandione] is a new formulation being developed for the M18 grenade.

[g] Brake fluid: DOT 4 is primarily composed of glycol and borate esters. DOT 5 is silicone-based brake fluid. The main difference is that DOT 4 is hydroscopic whereas DOT 5 is hydrophobic. DOT 5 is often used in military vehicles because it is more stable over time and requires less maintenance.

[h] Brake dust: Fe particles caused by abrasion of the cast iron brake rotor by the pad and secondly fibers from the semi-metallic elements of the brake pad. The remainder of the dust residue is carbon content within the brake pad.

[i] MIL-L-63460, "Military Specification, Lubricant, Cleaner and Preservative for Weapons and Weapons Systems;" trade name "Break-Free CLP" (http://www.midwayusa.com/product/1106170293/break-free-clp-bore-cleaning-solvent-lubricant-rust-preventative-liquid).

[j] High explosives: The M795 155 mm projectile is the U.S. Army/Marine Corp's current standard projectile containing 10.8 kg TNT. The M795 projectile replaced the M107 projectile that contained Composition B, which is a 60/40 mixture of RDX/TNT. RDX is cyclotrimethylene trinitramine. Suggestion: Test RDX/TNT together.

[k] Artillery propellant: Modern gun propellants are divided into three classes: single-base propellants, which are mainly or entirely nitrocellulose based; double-base propellants composed of a combination of nitrocellulose and nitroglycerin; and triple base composed of a combination of nitrocellulose and nitroglycerin and nitroguanidine. Suggestion: Test total nitrocellulose/nitroglycerin nitroguanidine together.