**AOAC SMPR 2016.XXX; Version 6**

**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 which 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.[1] 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.

[1] 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., Vol. 61, No. 1, pp. 91-95, 1989. Printed in Great Britain. @ 1989 IUPAC

**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 I.

**5. System Suitability Tests and/or Analytical Quality Control:**
The controls listed in Table II shall be embedded in assays as appropriate. Manufacturer must provide written justification if controls are not embedded in the assay.

**6. Validation Guidance:** AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures (AOAC INTERNATIONAL Official Methods of Analysis, 2012, Appendix I).

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.

**8. Maximum Time-to-Result**: Within four hours.

78 **Table I: Method Performance Requirements**

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| Parameter | Minimum Performance Requirement |
|---|---|
| AMDL | 2,000 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 III) must test positive at 2x the AMDL † |
| Exclusivity | All exclusivity strains (Table IV and Table V; part 2) must test negative at 10x the AMDL † |

Notes:

† 100% correct analyses are expected. All discrepancies are to be re-tested following the AOAC Guidelines for Validation of Biological Threat Agent Methods and/or Procedures[2].

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[2] Official Methods of Analysis of AOAC INTERNATIONAL (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, APPENDIX I; also on-line at http://www.eoma.aoac.org/app_i.pdf.

82 **TABLE II: Controls**

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| Control | Description | Implementation |
|---|---|---|
| **Positive Control** | This control is 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 Control** | This control is 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 Control** | This control is 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 III: 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 (USA) | 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 (S. 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 = The United States Army Medical Research Institute for Infectious Diseases.

**Table IV: 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$^{+b}$ |
| 15 | *B. cereus* subsp. *anthracis* | | |

[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.

**Guidance on Combining DNA for Exclusivity Evaluation**

DNA from exclusivity panel organisms 1 -9 in Table IV 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 re-tested at 10 times the AMDL.

DNA from exclusivity panel organisms 10 – 15 in Table IV can NOT be combined for exclusivity evaluation.

## Table V: 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 - the environmental organisms study; and part 3 - the potential Interferents applicable to Department of Defense applications.[3]

**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.

[3] Added in June 2015 for the Deprtment of Defense project.

**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 re-tested at 10 times the AMDL with and without the target gene or gene fragment at 2x 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[4]
  *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
  Gypsy moth cell lines LED652Y cell line (baculovirus)– Invitrogen
  Cockroach (commercial source)
  Tick (Amblyomma and *Dermacentor* tick species for *F. tularensis* detection assays)[5]
  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)[6]

- **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)

[4] If pollen is unavailable, vegetative DNA is acceptable
[5] Added by SPADA on (future approval date).
[6] Added by SPADA on September 1, 2015

## 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 VI 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 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 microgram per 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.

300 **Table VI: Potential Interferents**

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| **Compounds** | | **Potential Theaters of Operation** |
|---|---|---|
| group 1: petroleum-based | JP-8[1] | Airfield |
| | JP-5[2] | Naval |
| | diesel/gasoline mixture | Ground |
| | fog oil (standard grade fuel number 2) | naval, ground |
| | burning rubber[3] | ground, airfield |
| group 2: exhaust | gasoline exhaust | Ground |
| | jet exhaust | naval, airfield |
| | diesel exhaust | Ground |
| group 3: obscurants | terephthalic acid[4] | Ground |
| | zinc chloride smoke[5] | Ground |
| | solvent yellow 33[6] | Ground |
| group 4: environmental | burning vegetation | ground, airfield |
| | road dust | Ground |
| | sea water (sea spray) | Naval |
| group 5: chemicals | brake fluid[7] | All |
| | brake dust[8] | Ground |
| | cleaning solvent, *MIL-L-63460*[9] | All |
| | explosive residues<br>a) high explosives[10]<br>b) artillery propellant[11] | All |

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303 Table VI is offered for guidance and there are no mandatory minimum requirements for the
304 number of potential Interferents to be tested.

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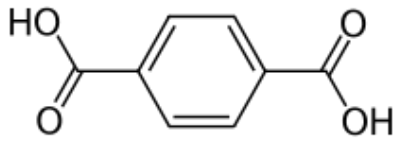
[1] **JP-8**. Air Force formulation jet fuel.

[2] **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.

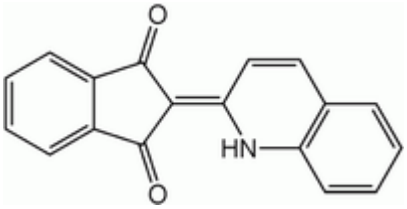
[3] **Burning rubber** (tire smoke). Gaseous C1-C5 hydrocarbons: methane; ethane; isopropene; butadiene; propane. Polycyclic aromatic hydrocarbons (58-6800 ng/$m^3$): parabenzo(a)pyrene; polychlorinated dibenzo-p-dioxins (PCDD); polychlorinated dibenzofurans (PCDF). Metals (0.7 - 8 mg/$m^3$): zinc; lead;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

[4] **Terephthalic acid.** Used in the AN/M83 hand grenade currently used by US military.




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

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




[7] **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 requires less maintenance

[8] **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.

[9] **MIL-L-63460**, "Military Specification, Lubricant, Cleaner and Preservative for Weapons and Weapons Systems”; trade name **“**Break-Free CLP**”.** Hyperlink: Midway USA.

[10] **High explosives**. The M795 155mm projectile is the US Army / Marine Corp's current standard projectile containing 10.8 kg of 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.

[11] **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.