Standard Method Performance Requirements (SMPRs) for Detection and Identification of Variola Virus

Intended Use: Laboratory use by trained technicians

1 Applicability

Detection of Variola virus DNA in collection buffers from aerosol collection devices for DoD applications.

Note: Method developers are advised to check the AOAC website for the most up to date version of this SMPR before initiating a validation.

2 Analytical Technique

Polymerase chain reaction (PCR) methods

3 Definitions

Acceptable minimum detection level (AMDL).—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). The AMDL is dependent on the intended use. (Draft EN ISO/CD 16140-1: Microbiology of food and animal feeding stuffs—Method validation—Part 1: Terminology of method validation, vs 17-03-2011)

Exclusivity. —Study involving pure nontarget strains that are potentially cross-reactive that shall not be detected or enumerated by the tested method. (Draft EN ISO/CD 16140-1: Microbiology of food and animal feeding stuffs—Method validation—Part 1: Terminology of method validation, vs 17-03-2011)

Inclusivity. —Study involving pure target strains that shall be detected or enumerated by the alternative method. (Draft EN ISO/CD 16140-1: Microbiology of food and animal feeding stuffs—Method validation—Part 1: Terminology of method validation, vs 17-03-2011)

4 System Suitability Tests and/or Analytical Quality Control

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

5 Validation Guidance

AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures (Official Methods of Analysis of AOAC INTERNATIONAL, 20th Ed., 2016, Appendix I).

6 Method Performance Requirements

See Table 1.

7 Maximum Time-to-Assay Result

≤4 h.

Approved by the AOAC Stakeholder Panel on Agent Detection Assays (SPADA). Final Version Date: September 1, 2016.

ANNEX I

Controls

See Table 2.

Table 1. Method performance requirements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Minimum performance requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceptable minimum detection level (AMDL)</td>
<td>50 000 copies/mL Variola virus target DNA in the candidate method sample collection buffer. Copies/mL refers to number of viral genomes or equivalent plasmid copies containing target viral gene or gene fragment.</td>
</tr>
<tr>
<td>Probability of detection at AMDL within sample collection buffer</td>
<td>≥0.95</td>
</tr>
<tr>
<td>Probability of detection at AMDL in an aerosol environmental matrix</td>
<td>≥0.95 (Annex V, Part 1)</td>
</tr>
<tr>
<td>Inclusivity panel purified DNA</td>
<td>All inclusivity strains (Annex II) must test positive at 2× the AMDL*</td>
</tr>
<tr>
<td>Exclusivity panel purified DNA</td>
<td>All exclusivity strains (Annex III and Annex V, Part 2) must test negative at 10× the AMDL*</td>
</tr>
<tr>
<td>System false-negative rate using spiked aerosol environmental matrix</td>
<td>≤5% (Annex V, Part 1)</td>
</tr>
<tr>
<td>System false-positive rate using aerosol environmental matrix</td>
<td>≤5% (Annex V, Part 1)</td>
</tr>
</tbody>
</table>

* 100% correct analyses are expected. All aberrations are to be retested following the AOAC INTERNATIONAL Methods Committee 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]. Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.
Table 2. Controls

<table>
<thead>
<tr>
<th>Control</th>
<th>Description</th>
<th>Implementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>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. It is recommended that a technique (i.e., unique distinguishable signature) is used to confirm whether the positive control is the cause of a positive signal generated by a sample.</td>
<td>Single use per sample (or sample set) run</td>
</tr>
<tr>
<td>Negative control</td>
<td>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.</td>
<td>Single use per sample (or sample set) run</td>
</tr>
<tr>
<td>Inhibition control</td>
<td>Designed to specifically address the impact of a sample or sample matrix on the assay’s ability to detect the target organism.</td>
<td>Single use per sample run</td>
</tr>
</tbody>
</table>

ANNEX II

Inclusivity Panel

The inclusivity panel shall include:

1. Sequences from at least two representative strains, one strain from each major clade of Variola virus [Li et al. (October 2, 2007) On the origin of smallpox: correlating Variola phylogenetics with historical smallpox records, PNAS 104(40), 15787–15792]
2. Any other strain with differences in the assay primer and/or probe target sequences based on bioinformatic analysis. See Annex IV.

Note: The World Health Organization (WHO) restricts access to Variola virus genomic material; use of any genomic sequences greater than 500 bp requires written permission/approval from the WHO. Insertion of Variola virus DNA into other Orthopoxviruses is prohibited.

More details can be found at:
WHO recommendations concerning the distribution, handling and synthesis of Variola virus DNA, http://www.who.int/csr/disease/smallpox/SummaryrecommendationsMay08.pdf

ANNEX III

Exclusivity Panel (Near Neighbor)

The exclusivity panel shall include:

1. All poxvirus strains listed in Table 3 (Note: See AOAC website for the most recent list.)
2. Any additional strains determined through the bioinformatics analysis, performed in accordance with Annex IV, with greater similarity to the assay’s target region(s) than the strains listed in Table 3.

ANNEX IV

Bioinformatics Analyses of Signature Sequences Underlying Variola Virus Assays

In silico screening will be performed on signature sequences (e.g., oligo primers) to demonstrate specificity to Variola virus and inclusivity across all sequenced Variola virus strains.

In silico results are suggestive of potential performance issues, so will guide necessary additions to the wet screening panels. In silico identification of potential cross-reactions (false positives) or nonverifications (false negatives) would require the affected strains be included in the exclusivity or inclusivity panels, respectively, if available.

A vendor-selected tool to carry out the bioinformatics evaluation should be able to predict hybridization events between signature components and a sequence in a database including available genomic sequence data, using public GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The selected tool should be able to identify predicted hybridization events based on platform annealing temperatures, thus ensuring an accurate degree of allowed mismatch is incorporated in predictions. The program should detect possible amplicons from any selected database of sequence.

Potential tools for in silico screening of real-time PCR signatures include:

1. http://sourceforge.net/projects/simulatepcr/files/?source=navbar. This program will find all possible amplicons and real-time fluorescing events from any selected database of sequence.
2. NCBI tools

The vendor submission should include:

1. Description of sequence databases used in the in silico analysis

<table>
<thead>
<tr>
<th>Table 3. Core exclusivity panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Vaccinia</td>
</tr>
<tr>
<td>Cowpox</td>
</tr>
<tr>
<td>Ectromelia</td>
</tr>
<tr>
<td>Monkeypox</td>
</tr>
<tr>
<td>Monkeypox</td>
</tr>
<tr>
<td>Racoonpox</td>
</tr>
<tr>
<td>Skunkpox</td>
</tr>
<tr>
<td>Volepox</td>
</tr>
<tr>
<td>Camelpox</td>
</tr>
<tr>
<td>Taterapox</td>
</tr>
<tr>
<td>Parapoxviruses Orf</td>
</tr>
</tbody>
</table>

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(2) Description of conditions used for in silico analysis. Stringency of in silico analysis must match bench hybridization conditions.

(3) Description of tool used for bioinformatics evaluation. Data demonstrating the selected tool successfully predicts specificity that has been confirmed by wet-lab testing on designated isolates. These data can be generated retrospectively using published assays.

(4) List of additional strains to be added to the inclusivity (Annex II) or exclusivity (Annex III) panels based on the bioinformatics evaluation

ANNEX 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. It is critical to understand the performance of the method in the presence of these environmental factors. This panel is used to characterize assay performance in the presence of these factors. 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

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

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

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Fungi:
  *Alternaria alternata*
  *Aspergillus fumagatus*
  *Aureobasidium pullulans*
  *Cladosporium cladosporioides*
  *Cladosporium sphaerospermum*
  *Epicoccum nigrum*
  *Eurotium amstelodami*
  *Mucor racemosus*
  *Paecilomyces variotii*
  *Penicillium 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™ 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)

**Capri hirca**
  (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 4 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 4 is offered for guidance, and there are no mandatory minimum requirements for the number of potential interferents to be tested.
## Table 4. Potential interferents

<table>
<thead>
<tr>
<th>Compound</th>
<th>Potential theaters of operation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: Petroleum-based</strong></td>
<td></td>
</tr>
<tr>
<td>JP-8a</td>
<td>Airfield</td>
</tr>
<tr>
<td>JP-5b</td>
<td>Naval</td>
</tr>
<tr>
<td>Diesel/gasoline mixture</td>
<td>Ground</td>
</tr>
<tr>
<td>Fog oil (standard grade fuel No. 2)</td>
<td>Naval, ground</td>
</tr>
<tr>
<td>Burning rubberc</td>
<td>Ground, airfield</td>
</tr>
<tr>
<td><strong>Group 2: Exhaust</strong></td>
<td></td>
</tr>
<tr>
<td>Gasoline exhaust</td>
<td>Ground</td>
</tr>
<tr>
<td>Jet exhaust</td>
<td>Naval, airfield</td>
</tr>
<tr>
<td>Diesel exhaust</td>
<td>Ground</td>
</tr>
<tr>
<td><strong>Group 3: Obscurants</strong></td>
<td></td>
</tr>
<tr>
<td>Terephthalic acidd</td>
<td>Ground</td>
</tr>
<tr>
<td>Zinc chloride smokee</td>
<td>Ground</td>
</tr>
<tr>
<td>Solvent yellow 33f</td>
<td>Ground</td>
</tr>
<tr>
<td><strong>Group 4: Environmental</strong></td>
<td></td>
</tr>
<tr>
<td>Burning vegetation</td>
<td>Ground, airfield</td>
</tr>
<tr>
<td>Road dust</td>
<td>Ground</td>
</tr>
<tr>
<td>Sea water (sea spray)</td>
<td>Naval</td>
</tr>
<tr>
<td><strong>Group 5: Chemicals</strong></td>
<td></td>
</tr>
<tr>
<td>Brake fluidg</td>
<td>All</td>
</tr>
<tr>
<td>Brake dusth</td>
<td>Ground</td>
</tr>
<tr>
<td>Cleaning solvent, MIL-L-63460i</td>
<td>All</td>
</tr>
</tbody>
</table>

Explosive residues: High explosives/ and artillery propellantj All

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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/m³): parabenzo(a)pyrene, polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF). Metals (0.7–8 mg/m³): 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 “HC smoke.” 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. A new formulation being develop for the M18 grenade.
g Brake fluid: DOT 4 is the most common brake fluid, 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.
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.
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.