AOAC SMPR 2014.006

Standard Method Performance Requirements for Detection and Identification of *Variola Virus* DNA in Aerosol Collection Filters and/or Liquids

Intended Use: Laboratory Use by Trained Technicians

1 Applicability

Detection and identification of *Variola virus* DNA in aerosol collection filters and/or liquids.

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).—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). The AMDL is dependent on the intended use. (Draft ISO 16140; 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, which are potentially cross-reactive, that shall not be detected or enumerated by the tested method. (Draft ISO 16140; *Ibid*)

Inclusivity.—Study involving pure target strains that shall be detected or enumerated by the alternative method. (Draft ISO 16140; *Ibid*)

Maximum time-to-assay result.—Maximum time to complete an analysis starting from the test portion preparation 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 [Appendix H: Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods, *Official Methods of Analysis of AOAC INTERNATIONAL*, 19th Ed., 2012].

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.

Variola virus.—A member of the genus *Orthopoxvirus* and the causative agent of smallpox.

Table 1. Method performance requirements

Parameter	Minimum performance requirement
Acceptable minimal detection level (AMDL)	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.
Probability of detection (POD) at AMDL within sample collection buffer	≥0.95
POD at AMDL in aerosol environmental matrix	≥0.95 (Annex IV, Part 1)
Inclusivity panel purified DNA	All inclusivity strains (Annex II and Annex V) must test positive at 2x the AMDL[a]
Exclusivity panel purified DNA	All exclusivity strains (Annex III and Annex IV, Part 2 and Annex V) must test negative at 10x the AMDL[a]
System false-negative rate using spiked aerosol environmental matrix	≤5% (Annex IV, Part 1)
System false-positive rate using aerosol environmental matrix	≤5% (Annex IV, Part 1)
Maximum time to assay result	24 h

[a] 100% correct analyses are expected. All aberrations are to be retested following the *AOAC Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures* [*Official Methods of Analysis of AOAC INTERNATIONAL* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Appendix I; also online at 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.

4 System Suitability Tests and/or Analytical Quality Control

The controls listed in Annex I shall be embedded in assays as appropriate. Manufacturers 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*, 19th Ed., 2012, Appendix I).

6 Other Requirements

Method developer must present the positive predictive value in their submission since smallpox is an eradicated disease. The positive predictive value must be based on data generated within the environmental matrix.

7 Method Performance Requirements

See Table 1.

Approved by AOAC Stakeholder Panel on Agent Detection Assays (SPADA). Final Version Date: August 14, 2014. Effective Date: October 15, 2014.

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 run

ANNEX I
Controls

See Table 2.

ANNEX II
Inclusivity Panel

The inclusivity panel shall include:

- Sequences from at least two representative strains from each major clade of *Variola virus*
- Any other strain with differences in the assay primer and/or probe target sequences based on bioinformatic analysis (Annex V).

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 http://www.who.int/csr/disease/smallpox/SummaryrecommendationsMay08.pdf

ANNEX III
Exclusivity Panel (Near-Neighbor)

The exclusivity panel shall include:

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

Table 3. Core exclusivity panel

Species	Strain	Commercial availability
Vaccinia	Elstree (Lister vaccine)	ATCC VR-1549
Cowpox	Brighton	ATCC VR-302
Ectromelia	Moscow	ATCC VR-1374
Monkeypox	V79-I-005	BEI Resources NR-2324
Monkeypox	USA-2003	BEI Resources NR-2500
Raccoonpox	Herman	ATCC VR-838
Skunkpox		ATCC
Volepox		ATCC
Camelpox		BEI Resources
Taterapox		BEI Resources
Parapoxvirus Orf	Vaccine	Colorado Serum Co.

ANNEX IV
Environmental Factors Panel for Validating PCR Detectors for Biothreat Agents

[Adapted from the environmental factors panel approved by SPADA on June 10, 2010. The working group determined that some of the environmental factors listed in the 2010 panel are not applicable to *Variola virus* detection assays, and so have been removed. Other various clarifications have been included. September 2014.]

The environmental factors panel is intended to supplement the biothreat agent near-neighbor exclusivity testing panel, and it should be applicable to all PCR biothreat agent detection assays. The panel criteria are divided into two main groups—the matrix panel of unknown environmental samples (Part 1); and the environmental panel of identified environmental organisms (Part 2). This panel will test for potential cross-reactive amplification and/or PCR inhibitors.

Part 1: Environmental Matrix Samples—Aerosol Environmental Matrices

Method developers should obtain environmental matrix samples that are representative and consistent with the collection method that is anticipated to be utilized in generating the sample being analyzed. 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.

- The aerosol environmental matrix pools should be tested with the target fragment at the AMDL to confirm the filter pool does not interfere with detection by the method used. Method developers will test the environmental matrix for interference with sufficient samples to achieve 95% POD.
- The aerosol environmental matrix pools should be used to confirm that there is no detection with the method used, i.e., there is no cross-reactivity of the target assay with unknown environmental organisms. 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 2x AMDL) and absence of the target viral gene or gene fragment. If an unexpected result occurs, each of the individual environmental organisms from a failed pool must be individually retested at 10x AMDL with and without the target viral gene or gene fragment at 4000 genome equivalents/mL in the candidate method DNA elution buffer.

- **Other Biothreat Agents**

  Bacillus anthracis Ames
  Yersinia pestis Colorado-92
  Francisella tularensis subsp. *tularensis* Schu-S4
  Burkholderia pseudomallei
  Burkholderia mallei
  Coxiella burnetii
  Brucella melitensis
  Ricinus communis (use ricin plant leaves as source of DNA)
  Clostridium botulinum Type A

- **Cultivatable Bacteria Identified as Being Present in Air and Soil**

  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 pneumophila
  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

- **DNA Viruses**

  Adenovirus vaccine
  Herpes simplex virus or *Cytomegalovirus* (whichever is available)

- **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**

  Plants.—
  Zea mays (corn)
  Pollen from *Pinus* spp. (pine)
  Gossypium hirsutum (cotton; use leaves from cotton plant as source of DNA)

  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)
  Gypsy moth cell lines LED652Y cell line (baculovirus; Invitrogen)
  Cockroach (commercial source)
  Tick (Amblyomma)

  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)

- **Biological Insecticides**

  Includes *Bacillus thuringiensis* subspecies that are widely used in agriculture. It is acknowledged that this organism is a near-neighbor of *B. anthracis* and has been included in the *B. anthracis* exclusivity panel. Furthermore, it is not closely related to *Y. pestis* and *F. tularensis*. However, 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)

  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)

ANNEX V
Bioinformatics Analyses of Signature Sequences Underlying *Variola Virus* Assays

In silico screening will be performed on signature sequences (e.g., oligo primers/probes) to predict 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 identify the relevant strains to be included in the exclusivity or inclusivity panels, respectively, if available.

A method developer-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 nucleotide [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:

- Simulate_PCR: 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.

- National Center for Biotechnology Information (NCBI) Toolbox: http://www.ncbi.nlm.nih.gov/IEB/ToolBox/index.cgi
- FastPCR: http://primerdigital.com/fastpcr.html

The method developer submission should include:

- Description of sequence databases used in the *in silico* analysis
- Description of tool used for bioinformatics evaluation

  Data demonstrating the selected tool successfully predicts specificity that has been confirmed by wet-laboratory testing on designated isolates.

  This data can be generated retrospectively using published assays.

- List of additional strains to be added to the inclusivity or exclusivity panels based on the bioinformatics evaluation