Method Name: Detection and Identification of Variola Virus

Approved Body: AOAC Stakeholder Panel on Agent Detection Assays

Approval Date:

Final version date:

1. Intended Use: Laboratory use by trained technicians.

2. Applicability: Detection and identification of Variola virus DNA in aerosol collection filters and/or liquids.


4. 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 with a probability of detection (POD) of 0.95 or higher. The AMDL is dependent on the intended use. (Draft ISO 16140) 

   Exclusivity
   Study involving pure non-target strains, which are potentially cross-reactive, that shall be not detected or enumerated by the tested method. (Draft ISO 16140)

   Environmental Interference
   Ability of the assay to be unaffected by environmental substances. Substances will be tested for cross reactivity and inhibition.

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

   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. (AOAC and Draft ISO).

   System False-Negative Rate
   Rate of negative system results contained within a population of known positive test portions.

   System False-Positive Rate

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1 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
5. **System suitability tests and/or analytical quality control:**
   The controls listed in Annex 1 shall be embedded in assays as appropriate. Manufacturer must provide written justification if controls are not embedded in the assay.

6. **Reference Material(s):**

7. **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).

8. **Maximum Time-To-Assay Result:** Maximum time to complete an analysis starting from the test portion preparation to assay result.
9. Method Performance Requirements:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Minimum Performance Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acceptable Minimal Detection Level (AMDL)</td>
<td>50,000 copies/ML of 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 @ AMDL within aerosol environmental matrix</td>
<td>0.95 or higher.</td>
</tr>
<tr>
<td>Inclusivity panel purified DNA</td>
<td>All inclusivity strains must test positive at 2x the AMDL†</td>
</tr>
<tr>
<td>Exclusivity panel purified DNA</td>
<td>All exclusivity strains must test negative at 10x the AMDL†</td>
</tr>
<tr>
<td>System False-Negative Rate within spiked aerosol environmental matrix</td>
<td>≤ 5%</td>
</tr>
<tr>
<td>System False-Positive Rate within aerosol environmental matrix</td>
<td>≤5%</td>
</tr>
<tr>
<td>Maximum Time to Assay Result</td>
<td>24 hours</td>
</tr>
</tbody>
</table>

Notes:
† 100% correct analyses are expected. All aberrations are to be re-tested following the AOAC Guidelines for Validation of Biological Threat Agent Methods and/or Procedures. Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

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## ANNEX I: Controls

<table>
<thead>
<tr>
<th>Control Type</th>
<th>Description</th>
<th>Usage</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive Control</strong></td>
<td>This control is designed to demonstrate an appropriate test response. This 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 the assay sensitivity is performing at previously determined level of sensitivity.</td>
<td>Single use per sample (or sample set) run</td>
<td>A positive control must perform as designed.</td>
</tr>
<tr>
<td><strong>Negative Control</strong></td>
<td>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.</td>
<td>Single use per sample (or sample set) run</td>
<td>A negative control must perform as designed.</td>
</tr>
<tr>
<td><strong>Inhibition Control</strong></td>
<td>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.</td>
<td>Single use per sample run</td>
<td>An inhibition control must perform as designed.</td>
</tr>
</tbody>
</table>
Annex II: Inclusivity Panel

The inclusivity panel should be designed based on bioinformatics analysis of the sequenced Variola virus strains in the assay target region. Sequences from at least two representative strains from each major clade of Variola virus should be included within the inclusivity panel as well as any other strain with differences in the assay primer and/or probe target sequences. 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. More details can be found at:

http://www.who.int/csr/disease/smallpox/SummaryrecommendationsMay08.pdf
Annex III: Exclusivity Panel (near-neighbor)

The exclusivity panel should be designed based on bioinformatics analysis of the assay target region. All poxvirus strains listed below should be included. In addition, any other strain of the poxvirus species below with greater similarity to your target region than the core exclusivity panel strain should be added to the exclusivity panel.

### CORE EXCLUSIVITY PANEL

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Commercial availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia</td>
<td>Elstree (Lister vaccine)</td>
<td>ATCC VR-1549</td>
</tr>
<tr>
<td>Cowpox</td>
<td>Brighton</td>
<td>ATCC VR-302</td>
</tr>
<tr>
<td>Ectromelia</td>
<td>Moscow</td>
<td>ATCC VR-1374</td>
</tr>
<tr>
<td>Monkeypox</td>
<td>V79-I-005</td>
<td>BEI NR-2324</td>
</tr>
<tr>
<td>Monkeypox</td>
<td>USA-2003</td>
<td>BEI NR-2500</td>
</tr>
<tr>
<td>Raccoonpox</td>
<td>Herman</td>
<td>ATCC VR-838</td>
</tr>
<tr>
<td>Skunkpox</td>
<td>SKP1991</td>
<td>ATCC (initial sample to be provided by CDC) x040224</td>
</tr>
<tr>
<td>Volepox</td>
<td>2004-CA-007</td>
<td>ATCC (initial sample to be provided by CDC) x040215</td>
</tr>
<tr>
<td>Camelpox</td>
<td>V78-I-2379</td>
<td>BEI (initial sample to be provided by CDC) x083899</td>
</tr>
<tr>
<td>Taterapox (gerbilpox)</td>
<td>V71-I-016</td>
<td>BEI (initial sample to be provided by CDC) x040170</td>
</tr>
<tr>
<td>Parapoxvirus Orf</td>
<td>vaccine</td>
<td>Colorado Serum Company</td>
</tr>
</tbody>
</table>
Annex IV: Environmental Factors Panel For Validating PCR Detectors For Biothreat Agents

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 and the environmental panel of identified environmental organisms. This panel will test for potential cross-reactive amplification as well as PCR inhibitors.

Environmental matrix samples - Aerosol Environmental matrices –
- The aerosol environmental matrix pools should be used to confirm there is not detection with vender candidate method i.e. there is no cross reactivity of the target assay with unknown environmental organisms.
- The aerosol environmental matrix pools should also be tested with the target fragment at the AMDL to confirm the filter pool does not interfere with detection by the vender candidate method.

- Vendors 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.
- Vendors will test the environmental matrix for interference with sufficient samples to achieve 95% probability of detection.
- Cross-reactivity testing will include sufficient samples and replicates to ensure each environmental condition is adequately represented.

Environmental Panel Organisms - This list is comprised of identified organisms from the environment. The organisms and cell lines will be tested as isolated DNA or pools of isolated DNA. It is acknowledged that some of these microorganisms are Select Agents. Inclusion of the entire panel of organisms included below is not necessary if the vendor can provide appropriate justification that the intended use of the assay permits the exclusion of specific items. A justification for any exclusion must be documented and provided.

- 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

- Cultivable bacteria identified as being present in air and soil

Acinetobacter lwoffii

Agrobacterium tumefaciens

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
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

Legionella pneumophila
Listeria monocytogenes
- **DNA Viruses**
  - *Adenovirus* vaccine
  - Herpes simplex or CMV – 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 recemosus*
  - *Paecilomyces variotii*
  - *Penicillium chrysogenum*
  - *Wallemia sebi*

- **DNA from higher eukaryotes**

- **Plants**
  - *Zea mays* (corn)
  - Pollen from *Pinus spp.* (pine)
  - Cotton – use leaves from cotton plant as source of DNA

- **Arthropods**
  - *Aedes aegypti* (ATCC /CCL-125) mosquito cell line
  - Mosquito (C6/36)* (Culex sp?)
  - Dust mite (commercial source)
  - Flea (Rocky Mountain labs)
  - *Drosophila cell line*
  - *Musca domestica* (housefly) ARS, USDA, Fargo, ND
  - Gypsy moth cell lines LED652Y cell line (baculovirus)– Invitrogen
  - Cockroach (commercial source)
  - Tick (Amblyoma )

- **Mammals**
  - *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) human
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 BA 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*

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)

The Environmental Panel Organism list as presented here was approved by SPADA on 12/13/07 and amended on 9/17/08.
Annex V: Bioinformatics Analyses of Signature Sequences underlying Variola Virus Assays

In silico screening will be performed on signature sequences (eg: 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 non-verifications (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 nt [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:

- [http://sourceforge.net/projects/simulatepcr/files/?source=navbar](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.

- NCBI tools

The vendor 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-lab 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