AOAC SMPR 2016.XXX; Version 3

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 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.

3. Analytical Technique: Polymerase Chain Reaction (PCR) Methods.

4. 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). The AMDL is dependent on the intended use. (Draft ISO 16140) [1]

Exclusivity
Study involving pure non-target strains, that are potentially cross-reactive, that shall not be detected or enumerated by the teste~~d~~ method. (Draft ISO 16140)[2]

Inclusivity
Study involving pure target strains that shall be detected or enumerated by the alternative method. (Draft ISO 16140)[3]

Maximum Time-To-Assay 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. [4] .

System False-Negative Rate

[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*
[2] *Ibid*.
[3] *Ibid*.
[4] Appendix H: Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods, Official Methods of Analysis of AOAC INTERNATIONAL, 19th edition, 2012.

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.

5. 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.

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).

64 **7. Method Performance Requirements**:

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Parameter	Minimum Performance Requirement
Acceptable Minimal Detection Level (AMDL)	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.
Probability of Detection at AMDL within sample collection buffer	≥ 0.95
Probability of Detection at AMDL in an aerosol environmental matrix	≥ 0.95 (Annex V; part 1)
Inclusivity panel purified DNA	All inclusivity strains (Annex II) must test positive at 2x the AMDL†
Exclusivity panel purified DNA	All exclusivity strains (Annex III and Annex V; part 2) must test negative at 10x the AMDL†
System False-Negative Rate using spiked aerosol environmental matrix	≤ 5% (Annex V; Part 1)
System False-Positive Rate using aerosol environmental matrix	≤ 5% (Annex V; Part 1)
Maximum Time to Assay Result	≤ 4 hours
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[5]. 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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- [5] 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.

67 **ANNEX I: 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. It is recommended that a technique (ie unique distinguishable signature) is used to confirm whether the positive control is the cause of a positive signal generated by a sample.	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 run

Annex II: Inclusivity Panel

The inclusivity panel shall include:

- Sequences from at least two representative strains, one strain from each major clade of *Variola virus (Li, et. al. On the origin of smallpox: correlating variola phylogenics with historical smallpox records. PNAS (2007) Oct. 2;104 (40):15787-15792.)*
- 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 Advisory Committee on Variola Virus Research: Report of the Seventeenth Meeting

Annex 5: WHO Recommendations concerning the distribution, handling and synthesis of variola virus DNA
http://apps.who.int/iris/bitstream/10665/205564/1/WHO_OHE_PED_2016.1_eng.pdf

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:

- All poxvirus strains listed in the table below (Note: See AOAC Website for the most recent list.)
- 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 the table below.

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 NR-2324
Monkeypox	USA-2003	BEI NR-2500
Raccoonpox	Herman	ATCC VR-838
Skunkpox	SKPV-USA-1978-WA	ATCC VR-1830
Volepox	VPXV-USA-1985-CA	ATCC VR-1831
Camelpox	V78-I-2379	BEI NR-49736 NR-49737
Taterapox	V71-I-016	BEI
Parapoxvirus Orf	vaccine	Colorado Serum Company

Annex IV: 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
  - 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 conditions used for *in silico* analysis
  - Stringency of *in silico* analysis must match bench hybridization conditions
- 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
- 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 - the environmental organisms study; and part 3 - the potential interferents applicable to Department of Defense applications.[6]

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.

[6] Added in June 2015 for the Department 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**

<u>Freshwater amoebae</u>
Acanthamoeba castellanii
Naegleria fowleri

<u>Fungi</u>
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[7]
  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)[8]

  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[9])

- **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)

[7] If pollen is unavailable, vegetative DNA is acceptable
[8] Added by SPADA on March 22, 2016.
[9] 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 1a 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 by 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.

337 **Table 5a: 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 a) high explosives[10] b) artillery propellant[11]	all

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340 Table 1a is offered for guidance and there are no mandatory minimum requirements for the number
341 of potential interferents to be tested.

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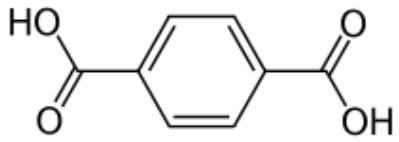
[1] **JP-8**. Air Force formulation jet fuel.

[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.

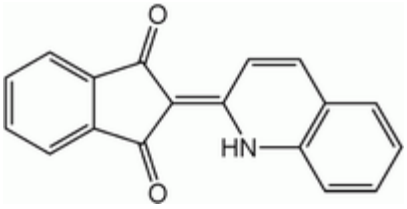
[3] **Burning rubber** (tire smoke). Gaseous C1-C5 hydrocarbons: methane; ethane; isopropene; butadiene; propane. Polycyclic aromatic hydrocarbons (58-6800 ng/m^3): parabenzo(a)pyrene; polychlorinated dibenzo-p-dioxins (PCDD); polychlorinated dibenzofurans (PCDF). Metals (0.7 - 8 mg/m^3): zinc; lead;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

[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.