AOAC SMPR® 2024.004

Standard Method Performance Requirements (SMPRs®) for Detection of Multiple Biothreat Agent Organisms in Environmental Samples by Amplicon Sequencing

Intended Use: Analysis of Aerosol Collection Dry Filter Unit (DFU) and/or Polytetrafluoroethylene (PTFE) Filters as Part of Concept of Operation-Based Routine Testing to Make Actionable Calls

1 Purpose

What: AOAC SMPRs® are voluntary consensus standards developed in accordance with the AOAC policy, "AOAC Due Process for Development of AOAC Non-Method Consensus Standards and Documents." SMPRs describe a scientific community's recommended minimum method performance characteristics and analytical requirements for a specific method-related intended use.

Who: Drafted by AOAC working groups, SMPRs are adopted by AOAC by a consensus of stakeholders affiliated with its integrated science programs and projects, which are composed of volunteer subject matter experts representing academia, government, industry, and nonprofit sectors from around the world.

Use: AOAC uses SMPRs in its core science programs in which they are a resource for AOAC method experts, including expert review panels, in the evaluation of validation study data for methods submitted to the AOAC *Official Methods of Analysis*℠ and AOAC *Performance Tested Methods*℠ programs. Additionally, AOAC SMPRs may be used to provide acceptance criteria for the verification of methods and serve as a resource to guide method development and optimization.

2 Applicability

Simultaneous detection of *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Brucella abortus*, *Brucella melitensis, Brucella suis, Coxiella burnetii*, and *Variola* virus from dry filter unit (DFU) filters or polytetrafluoroethylene (PTFE) filters. Suitable method may target some or all of these agents as appropriate for the specific biodefense program or concept of operations.

3 Analytical Technique

Amplicon sequencing of double stranded DNA-containing organisms only. This assay does not contain rRT-PCR assays that include a reverse transcription step.

4 Definitions

Acceptable minimum detection level (AMDL).—Predetermined minimum level of analyte, as specified by consensus, which must be detected by candidate method at specified probability of detection (POD).

Amplicon.—Segment of DNA that undergoes amplification and contains replicated genetic material.

Dry filter unit (DFU).—System used to collect aerosolized particulates on dry filter (typically 1 µm pore size, 3 mm thickness polyester felt, 47 mm in diameter) over period of time.

Environmental factors.—For the purposes of this SMPR: Any factor in operating environment of analytical method, whether abiotic or biotic, that might influence results of method.

Exclusivity.—Study involving pure nontarget strains that are potentially cross-reactive and should not be detected or enumerated by candidate method.

Inclusivity.—Study involving pure target strains that should be detected or enumerated by candidate method.

Interferent.—Substance in analytical procedures that, at given concentration, causes systematic error in analytical result (1). Sometimes also known as interferant.

Maximum time-to-result.—Maximum time to complete analysis starting from collection buffer to assay result.

Polytetrafluoroethylene (PTFE) filter.—Filter (typically 3 µm pore size, 47 mm diameter) sometimes used to collect aerosolized particulates.

Probability of detection (POD).—Proportion of positive analytical outcomes for qualitative method for given matrix at specified analyte level or concentration with ≥0.95 confidence interval.

System false-negative rate.—Proportion of test results that are negative contained within population of known positives. In the case of a validation study in which population of known positives consists of replicates of matrix at specified analyte concentration, system false-negative rate for that matrix is 1-POD at that concentration.

System false-positive rate.—Proportion of test results that are positive contained within population of known negatives. In the case of a validation study in which population of known negatives consists of replicates of matrix without analyte present, system false-positive rate for that matrix is POD at zero concentration.

Target.—Nucleic acid sequence intended to provide, in whole or in part, identity of organism or its characteristics.

Validation.—Establishment of performance characteristics of method and provision of objective evidence that performance requirements for specified intended use are fulfilled (2).

Verification.—Demonstration that validated method functions in user's hands according to method's specifications determined in validation study and is fit for its purpose (2).

5 Method Performance Requirements

See Table 1.

6 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blanks and appropriate quality control measures as described in Table 2. Manufacturer must provide written justification if controls are not embedded in method or assay. Controls should be representative of matrix type and serve as controls for entire process. For detailed discussion of appropriate quality controls, refer to Keenum et al. (3).

Sequencing and analysis workflow.—Analysis workflow for typical Oxford Nanopore Technologies-based amplicon sequencing experiment is described here. However, a similar strategy can be adopted for any other sequencing platform. In a sequencing experiment, one flow cell per 12 barcoded libraries [9 samples + 3 NTCs (no template controls)] is utilized. The 12 pooled libraries are loaded onto a flow cell and sequencing initiated via MinKNOW software. Base calling and demultiplexing (demux) are performed in real time using Guppy software. At user-defined time intervals (default 5 min), sample reads (Q score ≥10) are mapped to amplicon sequences stored in a local database and user-defined detection

thresholds are applied (*see* next section). Alignment metrics per amplicon are generated and false positives are calculated based on NTC alignments. Statistics are combined for amplicon and organism reports. Visualization and alignment count tables are downloadable at any time during analysis. When sequencing is finished or stopped after sufficient data for making actionable calls are collected, raw and processed data are compiled, stored, and encrypted as needed.

Detection thresholds.—The following thresholds are applied: *(1)* Per alignment threshold: Using BLASTN, ≥90% identity across ≥90% alignment length. These relaxed criteria are user-defined and can be adjusted depending on biothreat program or concept of operations requirements and data quantity and quality produced in specific run. There is also the assumption that in real-world wet lab testing, a small number of mismatches could still produce positive results in a sample.

(2) Per sample (barcode) threshold: Reference must have >2% of total aligned reads because it is assumed highly unlikely for a sample to be positive for >50 different targets.

(3) Per flow cell threshold: Based on NTC aligned read counts per amplicon. Amplicon "detection" will be masked in sample if sample aligned read count is < ($mean_{NTC}$ + 3 SD) aligned read count.

Criteria for target and organism calls.—To make actionable calls, multiple targets per organism may be required to achieve strain specificity. For example, *Bacillus anthracis* requires three targets for actionable call (*see* Table 3 for required targets per organism).

7 Validation Guidance

Validation is carried out according to OMA Appendix I (4) using the probability of detection (POD) model (5) for data analysis.

Method developers should make an effort to optimize method parameters to meet performance requirements in Table 1 when all target organisms are co-inoculated at the AMDL on filters. Any target organisms not detected at the AMDL under co-inoculation conditions should be validated under single inoculation conditions. Method claim statements or applicability statements should be clear as to which target organisms meet the SMPR under matrix co-inoculation conditions and which meet the SMPR only under matrix single inoculation conditions. For example, "Method X is applicable to the simultaneous detection of *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Burkholderia mallei*, and *Burkholderia pseudomallei*, and individual detection of *Brucella abortus* and *Variola* virus, on DFU filters according to AOAC SMPR 2024.004 criteria." Table 4 lists the type strains to be used for matrix testing.

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. Refer to OMA Appendix R (6). For each target agent, refer to required strains for inclusivity and exclusivity (near neighbors) in Tables 5–22. Refer to OMA Appendix O (7) for environmental panel organisms.

In silico analysis may be used to guide the wet lab inclusivity, exclusivity, and environmental organism testing. Report bioinformatics tool(s) used, conditions, and results of thermodynamic assessments of potential secondary structures, and results of sequence homologies between primers and organism DNA. Refer to OMA Appendix Q (8) and OMA Appendix T (9).

8 Maximum Time-to-Results

Maximum time for analysis of filters is 48 h.

Approved by stakeholders of the AOAC Stakeholder Program on Agent Detection Assays (SPADA). Final version: June 13, 2024. Effective date: July 1, 2024.

Co-funded by Joint Program Executive Office (JPEO), Joint Project Lead—Enabling Biotechnologies (CBRND), Defense Biological Product Assurance Office (DBPAO), and Department of Defense, Deputy Under Secretary of the Army, Test and Evaluation (DoD, DUSA TE).

Acknowledgments

AOAC Stakeholder Program on Agent Detection Assays Working Group for Amplicon Sequencing:

Shanmuga Sozhamannan (Chair)
Sharon Brunelle (AOAC Technical Consultant)
Sailaja Chandrapati
Wesley Colangelo
Michael Connolly
James E. Crill
Brett Forshey
Paul J. Jackson
Jonathan Jacobs
Ishi Keenum
Jason Kralj
Zhengfei Lu
Dev Mittar
Hiroki Nakae
Joyce Njoroge
Robert Player
Joseph Russell
J. Saravanan
Stephanie Servetas
Sanjiv Shah
Jennifer Stone
Michael Sussman
Lei Tang

Support from Deborah McKenzie (AOAC) and AOAC staff.

References

(1) Van Der Linden, W.E. (1989) *Pure Appl. Chem*. **61**, 91–95

(2) ISO 16140-1:2016 (2016) *Microbiology of the food chain—Method validation—Part 1: Vocabulary*, International Organization for Standardization, Geneva, Switzerland

(3) Keenum, I., Player, R., Kralj, J., Servetas, S., Sussman, M., Russell, J., Stone, J., Chandrapati, S., & Sozhamannan, S. (2023) *J. AOAC Int.* **106**, 1424–1430. https://doi.org/10.1093/jaoacint/qsad047

(4) *Official Methods of Analysis* (2023) Appendix I: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent Methods and/or Procedures*, George W. Latimer, Jr. (Ed), 22nd Ed., New York, NY, USA. https://doi.org/10.1093/9780197610145.005.009

(5) *Official Methods of Analysis* (2023) Appendix H: *Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods*, George W. Latimer, Jr. (Ed), 22nd Ed., New York, NY, USA. https://doi.org/10.1093/9780197610145.005.008

(6) *Official Methods of Analysis* (2023) Appendix R: *Guidelines for Verifying and Documenting the Relationships Between Microbial Cultures*, George W.

Latimer, Jr. (Ed), 22nd Ed., New York, NY, USA. https://doi.org/10.1093/9780197610145.005.018
(7) *Official Methods of Analysis* (2023) Appendix O: *Environmental Factors for Validating Biological Threat Agent Detection Assays*, George W. Latimer, Jr. (Ed) 22nd Ed., New York, NY, USA. https://doi.org/10.1093/9780197610145.005.015
(8) *Official Methods of Analysis* (2023) Appendix Q: *Recommendations for Developing Molecular Assays for Microbial Pathogen Detection Using Modern In Silico Approaches*, George W. Latimer, Jr. (Ed) 22nd Ed., New York, NY, USA. https://doi.org/10.1093/9780197610145.005.017
(9) *Official Methods of Analysis* (2023) Appendix T: *Standard Requirements for Nucleotide Sequences Used in Biothreat Agent Detection, Identification, and Quantification: Verified Next-Generation Sequences (VNGS)*, George W. Latimer, Jr. (Ed), 22nd Ed., New York, NY, USA. https://doi.org/10.1093/9780197610145.005.019
(10) Osterman, B., & Moriyon, I. (2006) *Int. J. Syst. Evol. Microbiol*. **56**, 1173–1175
(11) WHO Advisory Committee on Variola Virus Research: Report of the 17th Meeting: Annex 5: WHO Advisory Committee on Variola Virus Research. http://apps.who.int/iris/bitstream/10665/205564/1/WHO_OHE_PED_2016.1_eng.pdf
(12) WHO recommendations concerning distribution, handling, and synthesis of Variola virus DNA. https://www.who.int/publications/i/item/10665-241232
(13) Li et al. (2007) *PNAS* **104**, 15787–15792

ANNEX I:
Bioinformatics Analyses of Signature Sequences

Perform the following *in silico* analyses to demonstrate selectivity to target agents. *In silico* results are suggestive of potential performance issues, so results will guide the necessary wet lab testing. *In silico* identification of potential cross-reactions (false positives) or nonverifications (false negatives) would require affected strains be included in exclusivity or inclusivity panels, respectively, if available. *In silico* analysis cannot replace all wet lab testing but can serve to reduce the amount of wet lab testing required. Refer to OMA Appendix Q for more information (8).

Primary Sequence Alignments

Access known high-quality sequences for inclusivity and exclusivity organisms from trusted databases. Perform sequence alignments of genomes and primers.

Report inclusivity for each primer as number of genomes with perfect match sequence out of total number analyzed and report genomes with mismatches. For example, "Of the N target genomes analyzed, X were perfect matches for the forward primer. Those with mismatches were… The nature of the mismatches were…"

Report exclusivity as number of genomes with no matching sequences out of number of near neighbor genomes analyzed and report close matches where binding could occur. For example, "Of the N near neighbor genomes analyzed against the forward primer sequence, Y showed no matching sequences. Those showing potential for binding by the primer included…"

Report background selectivity as number of genomes with no matching sequences out of number of environmental genomes analyzed and report close matches where binding could occur. For example, "Of the N background genomes analyzed against the forward primer sequence, Z showed no matching sequences. Those showing potential for binding by the forward primer included…"

Unimolecular Folding Protocol

The purpose of performing thermodynamic folding simulations is to deduce if primers are able to bind to their targets without substantial unfolding of target. Primers that require substantial unfolding of target are often "fragile" and can give false negatives if mutation occurs at a primer binding site or if salt concentrations vary slightly (e.g., due to bad master mix lot or user intentionally diluting reagents). The steps below provide a recipe for determining quality of designs. The numbering referred to below is for the sense strand of the DNA virus. Use a program (e.g., MFOLD, VisualOMP, etc.) to predict the secondary structure of DNA target regions. An expert will evaluate reported results to deduce if primers are likely to have problems. To protect sensitive information, only amplicon positions need be reported, though if user does provide primer and probe sequences, then more specific guidance can be given about potential problems of the user's design.

(**a**) *Testing reverse primer (RP) binding region.—(1) Target DNA sequence*.—Parse out region where amplicon is made with extra 150 nucleotides on either side of amplicon (these extra regions are called 5′-and 3′-tail). For example, if amplicon covers positions 1000 to 1100 (i.e., amplicon length of 101 nucleotides), then DNA region to parse out would be from 850 to 1250 (i.e., 401 nucleotides long).

(2) Perform folding using "DNA" as strand type and use annealing temperature for PCR reaction (e.g., 50°C) and salt concentration representative of conditions in amplification reaction [suggested default values are (monovalent) = 0.08 M and (Mg^{2+}) = 0.002 M]. This is DG(total).

(a) RP region report.—Provide annealing temperature, salt conditions, DG(total), picture of secondary structure, and indicate positions where amplicon begins and ends.

(b) Optional.—Provide location of RP binding. If this optional information is provided, then more specific guidance can be given about potential problems of user's design.

(**b**) *Testing of forward primer (FP) binding region.—(1)* Make reverse complement of target region. This is DNA complementary strand to which forward primer will bind (which is referred to as cDNA target strand).

(2) Predict secondary structure of cDNA target region. Perform folding using "DNA" as strand type and use annealing temperature for PCR reaction (e.g., 60°C) and salt concentration representative of conditions in reverse transcription/PCR reaction [suggested default values are (monovalent) = 0.08 M and (Mg^{2+}) = 0.002 M]. This is DG(total).

(a) FP region report.—Provide annealing temperature, salt conditions, DG(total), picture of secondary structure, and indicate positions where amplicon begins and ends.

(b) Optional.—Provide location of FP binding. If this optional information is provided, then more specific guidance can be given about potential problems of user's design.

(**c**) *Primer unimolecular folding*.—For each primer, predict their secondary structure. Perform folding using "DNA" as strand type and use annealing temperature for PCR reaction (e.g., 60°C) and salt concentration representative of conditions in reverse transcription/PCR reaction [suggested default values are (monovalent) = 0.08 M and (Mg^{2+}) = 0.002 M]. This is DG(total).

(1) Primer folding report.—Provide annealing temperature, salt conditions, DG(total), and Tm.

(2) Optional.—Provide pictures of primer folding. If this optional information is provided, then more specific guidance can be given about potential problems of user's design.

(**d**) *Hybridization protocol.—(1)* Perform simulation of 2-state bimolecular hybridization for FP binding to the cDNA target [from step (**b**)(*1*)] under PCR salt conditions and using annealing temperature.

Report.—DG (annealing temperature), Tm (give strand concentrations used to make Tm prediction). If primer uses modified nucleotides, then provide best estimate of DG and Tm.

(2) Perform simulation of 2-state bimolecular hybridization for RP binding to DNA target [from step (**a**)(*1*)] under the PCR salt conditions and using annealing temperature.

Report.—DG (annealing temperature), Tm (give strand concentrations used to make Tm prediction). If primer uses modified nucleotides, then provide best estimate of DG and Tm.

Table 1. Method performance requirements

Parameter	Testing requirements	Performance criteria
Acceptable minimum detection level (AMDL)	Co-inoculation of all target organisms as spores or vegetative cells[a]	20,000 Genome equivalents per organism per filter
POD_{AMDL}	Target organisms at AMDL on filters	≥0.95 with 95% confidence
POD_0	Near neighbor at 10× AMDL and absence of target organisms on filters	≤0.05 with 95% confidence
Inclusivity[b]	2x AMDL	All targets must test positive[c]
Exclusivity[b]	10x AMDL	All must test negative

[a] Every effort should be made to optimize method to meet criteria for co-inoculation of all target organisms. For any organisms that fail co-inoculation testing, test by individual inoculation.
[b] Refer to Tables 5–22 for inclusivity and exclusivity panels.
[c] Known exceptions footnoted in Tables 5–22.

Table 2. Method quality controls[a]

Control	Description	Implementation
Positive	Designed to demonstrate appropriate test response. Positive control material should be included at low, but reliably detectable, concentration and should monitor performance of *all* molecular targets in panel. Purpose of using low concentration of positive control is to demonstrate that assays are performing at previously determined level of sensitivity. It is recommended that a technique (e.g., unique sequence tag) is used to confirm whether positive control is the cause of positive signal generated by a sample.	Must be analyzed for every sample or set of samples tested
Negative	Designed to demonstrate that amplicon sequencing panel does not produce any agent detection calls in absence of target organisms. Purpose of this control is to rule out causes of false positives, such as contamination.	Must be analyzed for every sample or set of samples tested
Inhibition	Designed to specifically address impact of sample or sample matrix on panel's ability to produce amplicon sequences. This may be performed in same reaction as target testing (e.g., by adding nontarget control to sample) or sample can be split into separate reactions for target(s) and control.	Must be analyzed for every sample tested

[a] Positive and negative controls should be representative of matrix type and serve as controls for entire process.

Table 3. Guidance on molecular targets for various biothreat agents

Agent	Required targets	Optional additional targets	Interpretation recommendations
Bacillus anthracis (BA)	*(1)* Target specific to BA chromosome *(2)* Target specific to pXO1 and pXO1-like plasmids *(3)* Target specific to pXO2 and pXO2-like plasmids	*(1)* Genes that contribute to virulence or drug-resistance *(2)* Regions for strain typing	–Detection of one or both plasmid targets but not chromosomal target does not conclusively indicate presence of BA as this may be caused by *B. cereus* biovar *anthracis* (example interpretation: BA *or* BC biovar *anthracis* detected) –Detection of BA-specific chromosomal targets but not one or both plasmid targets indicates presence of BA, but the BA may not be fully virulent (example interpretation: BA detected; presence of some virulence determinants not confirmed)
Francisella tularensis (FT)	Chromosomal target(s) inclusive of subsp. *tularensis* (Type A), *holarctica* (Type B), and *mediasiatica* that allows differentiation from *novicida* and other *Francisella* spp.	*(1)* Targets to distinguish between various subsp. *(2)* Target specific to *novicida* *(3)* Genes that contribute to virulence or drug-resistance *(4)* Regions for strain typing	–It is important to distinguish *novicida* from non-*novicida* FT as *novicida* is significantly less virulent, but often considered subsp. of FT rather than separate species (example interpretation: non-*novicida* FT subsp. detected)
Yersinia pestis (YP)	*(1)* Target specific to YP chromosome *(2)* Target specific to pPCP1 *(3)* Target specific to pMT1 *(4)* Target specific to pCD1 *(5)* Target specific to the high pathogenicity island (HPI)	*(1)* Genes that contribute to virulence or drug-resistance *(2)* Regions for strain typing	–Detection of only pCD1 does *not* indicate detection of YP as pCD1 is also found in other *Yersinia* spp. (example interpretation: YP not detected) –Detection of only some of non-PCD1 targets indicates presence of YP, but YP may not be fully virulent (example interpretation: YP detected; presence of some virulence determinants not confirmed)
Burkholderia mallei (BurkM) and *Burkholderia pseudomallei* (BurkP)	*(1)* Target specific to BurkM chromosome *(2)* Target specific to BurkP chromosome	*(1)* Genes that contribute to virulence or drug-resistance *(2)* Regions for strain typing	
Brucella spp.	*(1)* Target specific to *Brucella abortus* *(2)* Target specific to *Brucella melitensis* *(3)* Target specific to *Brucella suis*	*(1)* Targets inclusive of, or specific to, additional *Brucella* spp. *(2)* Genes that contribute to virulence or drug-resistance *(3)* Regions for strain typing	
Variola virus	Target specific to *Variola* virus	*(1)* Target to differentiate between *Variola major* and *Variola minor* *(2)* Targets inclusive of, or specific to, additional orthopox viruses (e.g., monkeypox, cowpox, camelpox, vaccinia, etc.) *(3)* Genes that contribute to virulence or drug-resistance *(4)* Regions for strain typing	
Coxiella burnetii	Target specific to *C. burnetii* chromosome	*(1)* Genes that contribute to virulence or drug-resistance *(2)* Regions for strain typing	

Table 4. Type strains to be used for matrix testing

Target organism	Type strain
Bacillus anthracis	Ames
Francisella tularensis	SCHU S4
Yersinia pestis	CO92
Burkholderia mallei	Strain 6
Burkholderia pseudomallei	1026b
Brucella abortus	S19
Brucella melitensis	16M
Brucella suis	1330
Variola virus	DNA of strain from Clade P-I
Coxiella burnetii	Nine Mile RSA439, Clone 4

Table 5. Inclusivity panel for *Bacillus anthracis*

No.	Cluster	Genotype	Strain	Origin	Characteristics[a]
1	A1a	7	Canadian bison	Wood bison	pXO1+, pXO2+, VNTR[b] genotype group A1a
2	A3a	45[c]	V770-NP-1R	Vaccine (United States)	pXO1+, pXO2–, VNTR genotype group A3A
3	A2	29	PAK-1	Sheep (Pakistan)	pXO1+, pXO2+, VNTR genotype group A2
4	A3a	51	BA1015	Bovine (Maryland)	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[d]	pXO1+, pXO2+, VNTR genotype group A4
10	B1	82	BA1035	Human (South 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[c]	Sterne	USAMRIID	pXO1+, pXO2–, VNTR genotype group A3b
14	A1b	23	Turkey No. 32	Human (Turkey)	pXO1+, pXO2+, VNTR genotype group A1b

[a] Inclusivity requirement: Chromosomal target plus at least one plasmid target must be positive.

[b] VNTR = Variable number tandem repeat.

[c] Organism contains only seven of eight multiple locus variable number tandem repeat analysis (MLVA) markers due to absence of pXO2. Genotypes listed are consistent with seven of the eight markers.

[d] USAMRIID = United States Army Medical Research Institute for Infectious Diseases.

Table 6. Exclusivity panel for *Bacillus anthracis*

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]

[a] pBCXO1 is pX01-like but not identical.
[b] capA, capB, and capC are contained within *Bacillus anthracis* pXO2 plasmid; however, capA, capB, and capC sequences are found in strains 03BB102 and 03BB108 in absence of pXO2 plasmid.

Table 7. Inclusivity panel for *Francisella tularensis*

No.	UCC[a] ID	Genus and species	Strain	Characteristics
1	FRAN001	*Francisella tularensis*	subsp. *tularensis*	Type A2 (Type strain)
2	FRAN004	*Francisella tularensis*	subsp. *holarctica* (LVS)	Type B (Russian)
3	FRAN012	*Francisella tularensis*	subsp. *holarctica*	Type B (United States)
4	FRAN016	*Francisella tularensis*	subsp. *tularensis* (SCHU S4)	Type A1 (United States)
5	FRAN024	*Francisella tularensis*	subsp. *holarctica* JAP (Cincinnati)	Type B (Japanese)
6	FRAN025	*Francisella tularensis*	subsp. *tularensis* (VT68)	Type A1 (United States)
7	FRAN029	*Francisella tularensis*	subsp. *holarctica* (425)	Type B (United States)
8	FRAN031	*Francisella tularensis*	subsp. *tularensis* (Scherm)	Type A1 (United States)
9	FRAN072	*Francisella tularensis*	subsp. *tularensis* (WY96)	Type A2 (United States)
10	NA	*Francisella tularensis*	subsp. *mediasiatica*	

[a] UCC = Department of Defense Unified Culture Collection; components available through Biodefense and Emerging Infections Research Resources Repository.

Table 8. Exclusivity panel for *Francisella tularensis*

No.	Species[a]	Strain
1	*Francisella philomiragia*	Jensen O#319L ATCC 25015
2	*Francisella philomiragia*	Jensen O#319-029 ATCC 25016
3	*Francisella philomiragia*	Jensen O#319-036 ATCC 25017
4	*Francisella philomiragia*	Jensen O#319-067 ATCC 25018
5	*Francisella philomiragia*	D7533, GA012794
6	*Francisella philomiragia*	E9923, GA012801
7	*Francisella novicida*	D9876, GA993548
8	*Francisella novicida*	F6168, GA993549
9	*Francisella novicida*	U112, GA993550
10	*Francisella hispaniensis*	DSM 22475

[a] *Francisella novicida* is sometimes considered to be subsp. of *F. tularensis*, but one or more targets should be used that allow differentiation between the two species.

Table 9. Inclusivity panel for *Yersinia pestis*

No.	Strain	Achtman genotype	Comment[a,b]	Availability[c]
1	CO92	1.ORI.c	Well-studied example of epidemic strain of pestis, recent isolate	CDC, USAMRIID
2	KIM	2.Med	Well-studied strain in academic circles, virulence data extensive	CDC, USAMRIID
3	Antiqua	1.Ant b	Ancient strain near root of tree	CDC, USAMRIID
4	Pestoides B	0.PE1		CDC, USAMRIID
5	Pestoides F	0.PE2.a	pPst negative, old strain in terms of phylogeny	CDC, USAMRIID
6	Pestoides G	0.PE2.b	pPst negative	CDC, USAMRIID
7	Angola	0.PE3	A “pestoides” in everything except name	CDC, USAMRIID
8	Nairobi	1.Ant a		CDC, USAMRIID
9	Harbin35	2 Ant	Rumored to be used or resulted from infection during experiments by Japanese BW Unit 731	CDC, USAMRIID
10	PBM19	1.ORI.a		CDC, USAMRIID
11	Java9	1.ORI	pFra negative	CDC, USAMRIID
12	A1122	1.ORI.a	Well-characterized U.S. isolate that is pgm- and pCD-; also has 2X large pPst plasmid	CDC, USAMRIID
13	Nicholisk 41	2.ANT		CDC, USAMRIID
14	Shasta	1.ORI	YE0387; Shasta (20 Oct 54); Shasta; human case; USA: Ca; 1960 6LY; UCC YERS074	CDC, USAMRIID
15	Dodson	1.ORI	Dodson (Aug 70); human case: male age 4.5 years; USA: Arizona (Tuba City); 27 Jun 67; UCC YERS073	CDC, USAMRIID
16	El Dorado			

[a] Note on plasmid nomenclature: pMT1 = pFra; pPCP1 = pPst = pPla; pCD1 = pYV= pCad.
[b] Inclusivity requirement: Chromosomal target and any plasmid targets corresponding to plasmids that are known to be carried by tested strain (not all strains contain all plasmids).
[c] CDC = Centers for Disease Control and Prevention; USAMRIID = U.S. Army Medical Research Institute of Infectious Diseases.

Table 10. Exclusivity panel for *Yersinia pestis*

No.	Species	Strain		Comment[a]	Availability[b]
1	*Yersinia ruckeri*	YERS063			USAMRIID
2	*Yersinia rohdei*	YERS062			USAMRIID
3	*Yersinia pseudotuberculosis*	PB1/+	1	Sequenced	WRAIR
4	*Yersinia pseudotuberculosis*	IP32953	1	Sequenced	WRAIR
5	*Yersinia pseudotuberculosis*	YPIII	3	Sequenced	WRAIR
6	*Yersinia pseudotuberculosis*	Pa3606	1b		WRAIR
7	*Yersinia pseudotuberculosis*	IB	1b		WRAIR
8	*Yersinia pseudotuberculosis*	EP2/+	1		WRAIR
9	*Yersinia pseudotuberculosis*	MD67	1		WRAIR
10	*Yersinia pseudotuberculosis*	1	1a		WRAIR
11	*Yersinia enterocolitica*	WA	O:8		WRAIR
12	*Yersinia enterocolitica*	8081	O:8	Sequenced	WRAIR
13	*Yersinia enterocolitica*	2516-87	O:9		WRAIR
14	*Yersinia kirstensenii*	Y231		Nonpathogenic	WRAIR
15	*Yersinia frederiksenii*	Y225		Nonpathogenic	WRAIR
16	*Yersinia intermedia*	Y228		Nonpathogenic	WRAIR
17	*Yersinia aldovae*	670-83		Nonpathogenic	WRAIR

[a] Exclusivity requirement: No detection of any *Y. pestis* targets *except* pCD1 (if applicable); pCD1 is not specific to *Y. pestis*, and this plasmid may be present in exclusivity panel members.
[b] USAMRIID = U.S. Army Medical Research Institute of Infectious Diseases; WRAIR = Walter Reed Army Institute of Research.

Table 11. Inclusivity panel for *Brucella abortus*[a]

No.	Strain designation	Biovar	ATCC/BEI/Accession No.	Available from	Comment
1	*B. abortus* S19	1	NR-10134	NVSL	S19 vaccine strain, smooth
2	*B. abortus* RB51	1	BEI NR-2552	NVSLBEI Resources	RB51 vaccine strain, rough
3	*B. abortus* 86/8/59	2	ATCC 23449BEI NR-231	BEI Resources	Bovine, England
4	*B. abortus* 12	3	ATCC 17385BEI NR-229	BEI Resources	
5	*B. abortus* Tulya	3	ATCC 23450BEI NR-232	BEI Resources	Human, Uganda
6	*B. abortus* 292 (39/94)	4	ATCC 23451BEI NR-233	BEI Resources	Bovine, England
7	*B. abortus* B3196	5	ATCC 23452BEI NR-234	BEI Resources	Bovine, England
8	*B. abortus* 870	6	ATCC 23453BEI NR-261	BEI Resources	Bovine, Africa
9	*B. abortus* 63/75	7	ATCC 23454BEI NR-262	BEI Resources	Bovine, Africa
10	*B. abortus* C68	9	ATCC 23455BEI NR-263	BEI Resources	Bovine, England
11	*B. abortus* 544	1	ATCC 23448BEI NR-69	BEI Resources	Bovine, England

[a] Add these strains to exclusivity panels for *B. melitensis* and *B. suis.*

Table 12. Inclusivity panel for *Brucella melitensis*[a]

No.	Strain designation	Biovar	ATCC/BEI/Accession No.	Available from	Comment
1	*B. melitensis* 16M	1	ATCC 23456 BEI NR-256	BEI Resources	Goat, USA
2	*B. melitensis* 63/9	2	ATCC 23457CP007789CP007788 BEI NR-257	Not commercially available in the U.S. at this time	Goat, Turkey
3	*B. melitensis Ether*	3	ATCC 23458 BEI NR-258	BEI Resources	Goat, Italy
4	*B. melitensis* bv. 1 str. Rev. 1	1	ACEG00000000	Not commercially available in the U.S. at this time	Elberg origin, *B. melitensis* vaccine strain

[a] Add these strains to exclusivity panels for *B. abortus* and *B. suis*.

Table 13. Inclusivity for *Brucella suis*[a]

No.	Strain designation	Biovar	ATCC/BEI/Accession No.	Available from	Comment
1	*B. suis* 1330	1	ATCC 23444 BEI NR-302	BEI Resources	Swine, USA
2	*B. suis Thomsen*	2	ATCC 23445 BEI NR-303	BEI Resources	Hare, Denmark
3	*B. suis* 686	3	ATCC 23446 BEI NR-304	BEI Resources	Swine, USA
4	*B. suis* 40	4	ATCC 23447 BEI NR-305	BEI Resources	Reindeer, Russia
5	*B. suis* 513	5	ACBK00000000	GenBank	Mouse, Russia
6	*B. suis* S2	NA	ALOS00000000.1	GenBank	Naturally attenuated vaccine strain used in China

[a] Add these strains to exclusivity panels for *B. melitensis* and *B. abortus.*

Table 14. Exclusivity panel for *Brucella abortus, B. melitensis*, and *B. suis*

No.	Strain designation	Biovar	ATCC/BEI/Accession No.	Available from	Comment
1	*B. canis* RM-666	NA	ATCC 23365 NR-683	ATCC	Dog
2	*B. neotomae* 5K33	NA	ATCC 23459 BEI NR-684	ATCCBEI Resources	Desert Wood Rat
3	*B. ovis* 63-390	NA	ATCC 25840 BEI NR-682	ATCCBEI Resources	Ram, Australia
4	*B. ceti* B1/94	NA	AZBH02000000	Not commercially available in the U.S. at this time	Porpoise, Scotland
5	*B. pinnipedialis* B2/94	NA	ACBN00000000	Not commercially available in the U.S. at this time	Seal, Scotland
6	*Brucella* spp. 83/13	NA	ACBQ00000000	Not commercially available in the U.S. at this time	Rat, Australia
7	*B. inopinata* BO1	NA	ADEZ00000000	Not commercially available in the U.S. at this time	Human, Oregon
8	*Brucella* sp. BO2	NA	ADFA00000000	Not commercially available in the U.S. at this time	Human, Australia
9	*B. papionis* F8/08-60(T)	NA	ACXD00000000	Not commercially available in the U.S. at this time	Novel Brucella associated with primates (NVSL 07-0026)
10	*B. microti* CCM 4915	NA	CP001578CP001579	Not commercially available in the U.S. at this time	Vvole, Czech Republic
11	*B. vulpis*	NA	LN997863-LN997864	Not commercially available in the U.S. at this time	Red fox, Austria
12	*Agrobacterium tumefaciens*	NA	ATCC 4452	ATCC	
13	*Ochrobactrum anthropi*	NA	ATCC 49188	ATCC	
14	*Ochrobactrum intermedium* LMG 3301	NA	SAMN02472089		

Note 1: The AOAC SPADA Working Group on *Brucella* is aware that *B. canis* can infect humans, causing approximately 100 cases of human brucellosis annually. The working group is also aware of the close relationship between *B. suis* and *B. canis.* In fact, the taxonomic classification of all *Brucella* spp. has undergone debate during the last few decades, with some scientists proposing that all *Brucella* spp. should be reclassified as *B. melitensis* on the basis of results of DNA-DNA hybridization and that the current species should be reclassified as biovars. However, the classic taxonomic scheme for *Brucella* spp. and existing biovars was reapproved in 2003 (10) on the basis of host specificity, phenotypic characteristics, varying virulence, and genotyping data. For these reasons as well as directions from DoD to focus on *B. suis,* the working group determined to develop this SMPR for the specific detection of *B. suis*.

Note 2: The AOAC SPADA Working Group on *Brucella* is aware of Russian vaccines using *B. abortus* SR82 and *B. abortus* 7579, and other strains may also be in use. These vaccine strains were not available at the time this SMPR was adopted. Consequently, the working group decided not to include these vaccine strains in the exclusivity panel.

Note 3: Available in the whole genome database at GenBank.

Table 15. Inclusivity panel for *Burkholderia pseudomallei*

Species	Isolate	Available from	Comment
B. pseudomallei	MSHR668BEI NR-9922	BEI Resources	Clinical Australian isolate
B. pseudomallei	MSHR1655		Clinical Australian isolate DBPAO[a]
B. pseudomallei	K96243BEI NR-4073	BEI Resources	Clinical Thai isolate
B. pseudomallei	MSHR305BEI NR-44225	BEI Resources	Clinical Australian isolate
B. pseudomallei	1026bBEI NR-9910BEI NR-4074	BEI Resources	Clinical Thai isolate
B. pseudomallei	7894		DBPAO
B. pseudomallei	MSHR840		Clinical Australian isolate DBPAO
B. pseudomallei	576aBEI NR-9916	BEI Resources	Clinical Thai isolate
B. pseudomallei	HBPUB10134aBEI NR-44220	BEI Resources	Clinical Thai isolate
B. pseudomallei	RF80		Environmental isolate from Thailand

[a] DBPAO = Defense Biological Products Assurance Office.

Table 16. Exclusivity panel for *Burkholderia pseudomallei*

No.	Species	Isolate
1	*B. mallei*	Strain 6 NCTC 10248 BEI NR-36126
2	*B. mallei*	China 5 BEI NR-21
3	*B. thailandensis*	CDC3015869 (TXDOH)
4	*B. thailandensis*	H0587
5	*B. thailandensis*	Malaysia20
6	*B. thailandensis*	E1
7	*B. humptydooensis* (proposed)	MSMB43 ATCC BAA-2767
8	*B. humptydooensis* (proposed)	MSMB1589
9	*Burkholderia* species MSMB264	MSMB0265
10	*B. oklahomensis*	1974002358
11	*B. oklahomensis*-like	BDU8
12	*Burkholderia* species MSMB175	TSV85
13	*B. ubonensis*	MSMB2036
14	*B. ubonensis*	MSMB1189
15	*B. multivorans*	AU1185
16	*B. stagnalis*	MSMB735
17	*B. cepacia (B. cenocepacia)*	MSMB1824
18	*B. vietnamiensis*	FL-2-3-30-S1-D0
19	*B. vietnamiensis*	AU1233

Table 17. Inclusivity panel for *Burkholderia mallei*

No.	Species	Isolate
1	*B. mallei*	Strain 6 NCTC 10248 BEI NR-36126
2	*B. mallei*	China 5 BEI NR-21
3	*B. mallei*	2002734306, NCTC120
4	*B. mallei*	2002734299, NCTC10229
5	*B. mallei*	2002734300, NCTC10247
6	*B. mallei*	China 7
7	*B. mallei*	2002734317, NCTC3709 (strain 106)
8	*B. mallei*	2000031063
9	*B. mallei*	2000031064, India 86-567-2
10	*B. mallei*	2002721276, KC237
11	*B. mallei*	2002721280, KC 1092, 52-236-Pasteur Institute

Table 18. Exclusivity panel for *Burkholderia mallei*

No.	Species	Isolate	Available from	Comment
1	*B. pseudomallei*	MSHR668BEI NR-9922	BEI Resources	Clinical Australian isolate
2	*B. pseudomallei*	MSHR1655		Clinical Australian isolate DBPAO[a]
3	*B. pseudomallei*	K96243BEI NR-4073	BEI Resources	Clinical Thai isolate
4	*B. pseudomallei*	MSHR305BEI NR-44225	BEI Resources	Clinical Australian isolate
5	*B. pseudomallei*	1026bBEI NR-9910BEI NR-4074	BEI Resources	Clinical Thai isolate
6	*B. pseudomallei*	7894		DBPAO
7	*B. pseudomallei*	MSHR840		Clinical Australian isolate DBPAO
8	*B. pseudomallei*	576aBEI NR-9916	BEI Resources	Clinical Thai isolate
9	*B. pseudomallei*	HBPUB10134aBEI NR-44220	BEI Resources	Clinical Thai isolate
10	*B. pseudomallei*	RF80		Environmental isolate from Thailand
11	*B. thailandensis*	CDC3015869 (TXDOH)		
12	*B. thailandensis*	H0587		
13	*B. thailandensis*	Malaysia20		
14	*B. thailandensis*	E1		
15	*B. humptydooensis* (proposed)	MSMB43 ATCC BAA-2767		
16	*B. humptydooensis* (proposed)	MSMB1589		
17	*Burkholderia* species MSMB264	MSMB0265		
18	*B. oklahomensis*	1974002358		
19	*B. oklahomensis*-like	BDU8		
20	*Burkholderia* species MSMB175	TSV85		
21	*B. ubonensis*	MSMB2036		
22	*B. ubonensis*	MSMB1189		
23	*B. multivorans*	AU1185		
24	*B. stagnalis*	MSMB735		
25	*B. cepacia (B. cenocepacia)*	MSMB1824		
26	*B. vietnamiensis*	FL-2-3-30-S1-D0		
27	*B. vietnamiensis*	AU1233		

Table 19. Inclusivity panel for *Variola* virus[a,b]

Clade[c]	No. Strains	Comments
P-I	1	Choose any strain from P-I
P-II	1	Choose any strain from P-II
Any	As needed based on bioinformatics[d]	Test all strains with differences in assay primer and/or probe target sequences

[a] Test using synthetic DNA sequences.
[b] 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. *See* refs. 11 and 12.
[c] *See* ref. 13.
[d] *See* Annex I.

Table 20. Exclusivity panel for *Variola* virus[a]

No.	Species	Strain	Commercial availability
1	*Vaccinia*	Elstree (Lister vaccine)	ATCC VR-1549
2	*Cowpox*	Brighton	ATCC VR-302
3	*Ectromelia*	Moscow	ATCC VR-1374
4	*Monkeypox*	V79-I-005	BEI NR-2324
5	*Monkeypox*	USA-2003	BEI NR-2500
6	*Raccoonpox*	Herman	ATCC VR-838
7	*Skunkpox*	SKPV-USA-1978-WA	ATCC VR-1830
8	*Volepox*	VPXV-USA-1985-CA	ATCC VR-1831
9	*Camelpox*	V78-I-2379	BEI NR-49736
10	*Taterapox*	V71-I-016	BEI NR-49737
11	*Parapoxvirus Orf*	Vaccine	Colorado Serum Co.

[a] In addition to poxvirus strains listed in Table 19, also test any additional strains determined through bioinformatics to have greater similarity to assay's target region(s) than strains listed here. *See* Annex I.

Table 21. Inclusivity panel for *Coxiella burnetii*

Phylogenetic group	Isolate (example)
1	Nine Mile RSA493 Nine Mile RSA439
2	Henzerling
3	C
4	K
5	G
6	Dugway

Table 22. Exclusivity panel for *Coxiella burnetii*

No.	Species	Strain
1	*Legionella pneumophila*	Philadelphia 1
2	*Legionella pneumophila*	Wadsworth 1
3	*Legionella pneumophila*	Sg6
4	*Legionella longbeachae*	ATCC No. 33462
5	*Rickettsiella* spp.	If obtainable