

Standard Method Performance Requirements for Immunological-Based Handheld Assays (HHAs) for Detection of *Bacillus anthracis* Spores in Visible Powders

Intended Use: Field use by first responders for analysis of visible powders

Method Developer and Independent Validation Studies

Probability of Detection at the Acceptable Minimum Detection Level

1 Definitions

Probability of detection (POD) is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given agent level or concentration. POD is concentration-dependent. The acceptable minimum detection level (AMDL) is the predetermined minimum level of a biological threat agent, which must be detected by the candidate method with an estimated 5% lower confidence limit on the POD of 0.95 or higher. The AMDL is dependent on the intended use.

2 Test Conditions

AMDL is 10^7 CFU/mL *Bacillus anthracis* Ames spores in candidate method sample collection buffer.

3 Acceptance Criteria

Estimated 5% lower confidence limit on the POD must be 0.95 or higher. (No more than one failure in 96 replicates.)

Inclusivity

1 Definition

Strains or isolates or variants of the target agent(s) that the method can detect (Table 1).

2 Test Conditions

Test spores of each member of the *Bacillus anthracis* inclusivity panel at AMDL.

3 Acceptance Criteria

100% positive results.

Note: In the case of a negative result, retest that strain 96 times with no failures allowed to demonstrate an estimated 5% lower confidence limit on the POD of 0.95 or higher.

Exclusivity

1 Definition

Nontarget agents, which are potentially cross-reactive, that are not detected by the method (Table 2).

2 Test Conditions

Test spores of each member of the *Bacillus anthracis* HHA exclusivity panel at 10 times AMDL.

3 Acceptance Criteria

100% negative results.

Table 1. *Bacillus anthracis* HHA method: Inclusivity panel

No.	Cluster	Genotype	Strain	MRI No. ^a	Origin	Characteristics
BA1	A1a	7	Canadian bison	107448	Wood bison	pX01+, pX02+, VNTR genotype group A1a
BA2	A3a	45 ^b	V770-NP-1R	107240	Vaccine (USA)	pX01+, pX02-, VNTR genotype group A3a
BA3	A2	29	PAK-1	107518	Sheep (Pakistan)	pX01+, pX02+, VNTR genotype group A2
BA4	A3a	51	BA1015	107446	Bovine (MD)	pX01+, pX02+, VNTR genotype group A3a
BA5	A3b	62	Ames	107517	Bovine (Texas)	pX01+, pX02+, VNTR genotype group A3b
BA6	A3c	67	K3	107497	South Africa	pX01+, pX02+, VNTR genotype group A3c
BA7	A3d	68	Ohio ACB	107339	Pig	pX01+, pX02+, VNTR genotype group A3d
BA8	A4	69	SK-102 (Pakistan)	107449	Imported wool (Pakistan)	pX01+, pX02+, VNTR genotype group A4
BA9	A4	77	Vollum 1B	107539	USAMRIID ^a	pX01+, pX02+, VNTR genotype group A4
BA10	B1	82	BA1035	107451	Human (South Africa)	pX01+, pX02+, VNTR genotype group B1
BA11	B2	80	RA3	107520	Bovine (France)	pX01+, pX02+, VNTR genotype group B2
BA12	C	Unk ^c	2002013094 (240)	124030	Louisiana	pX01+, pX02+, VNTR genotype group C
BA13	A1a	8	Pasteur	107171	USAMRIID	pX01-, pX02+, VNTR genotype group A1a
BA14	A3b	59, 61 ^b	Sterne	107453	USAMRIID	pX01+, pX02-, VNTR genotype group A3b
BA15	A1b	23	Turkey No. 32	107255	Human (Turkey)	pX01+, pX02+, VNTR genotype group A1b

^a MRI = MRI Global; USAMRIID = The United States Army Medical Research Institute for Infectious Diseases.

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^b Organism contains only seven of eight MLVA markers due to the lack of pX02. Genotypes listed are consistent with seven of the eight markers. (*Note:* Footnote applies to BA2 and BA14 genotype designations.)

^c Unk = Unknown.

Table 2. *Bacillus anthracis* HHA method: Exclusivity panel

No.	Species	Strain	Plasmid status
BANN1	<i>B. cereus</i>	S2-8	pXO1-, pXO2-
BANN2	<i>B. cereus</i>	3A	pXO1-, pXO2-
BANN3	<i>B. thuringiensis</i>	HD1011	pXO1-, pXO2-
BANN4	<i>B. thuringiensis</i>	97-27	pXO1-, pXO2-
BANN5	<i>B. thuringiensis</i>	HD682	pXO1-, pXO2-
BANN6	<i>B. cereus</i>	E33L	pXO1-, pXO2-
BANN7	<i>B. cereus</i>	D17	pXO1-, pXO2-
BANN8	<i>B. thuringiensis</i>	HD571	pXO1-, pXO2-
BANN9	<i>B. cereus</i>	Al Hakam	pXO1-, pXO2-
BANN10	<i>B. cereus</i>	ATCC 4342	pXO1-, pXO2-
BANN11	<i>B. cereus</i>	FM1	pXO1-, pXO2-
BANN12	<i>B. cereus</i>	G9241	pBCXO1 ^a , pXO2-
BANN13	<i>B. cereus</i>	03BB102	pXO1+, capA+, capB+, capC ^b
BANN14	<i>B. cereus</i>	03BB108	pXO1+, capA+, capB+, capC ^b
BANN15	<i>B. thuringiensis</i>	subsp. <i>israelensis</i> HD 1002	pXO1-, pXO2-
BANN16	<i>B. thuringiensis</i>	subsp. <i>kurstaki</i> HD 1	pXO1-, pXO2-
BANN17	<i>B. thuringiensis</i>	subsp. <i>morrisoni</i> HD 600	pXO1-, pXO2-
BANN18	<i>B. coagulans</i>	ATCC 7050	pXO1-, pXO2-
BANN19	<i>B. mycoides</i>	ATCC 6462	pXO1-, pXO2-
BANN20	<i>B. megaterium</i>	ATCC 14581	pXO1-, pXO2-
BANN21	<i>B. cohnii</i>		pXO1-, pXO2-
BANN22	<i>B. psychrosaccharolyticus</i>		pXO1-, pXO2-
BANN23	<i>B. benzoovorans</i>		pXO1-, pXO2-
BANN24	<i>B. megaterium</i>		pXO1-, pXO2-
BANN25	<i>B. orikoshii</i>		pXO1-, pXO2-
BANN26	<i>B. macroides</i>		pXO1-, pXO2-
BANN27	<i>B. clausil</i>	DSM 8716T	
BANN28	<i>B. vedderi</i>	DSM 9768T	
BANN29	<i>B. subtilis</i>	ATCC 6051T	
BANN30	<i>B. licheniformis</i>		
BANN31	<i>B. amyloliquefaciens</i>		
BANN32	<i>B. circulans</i>		
BANN33	<i>B. pumilus</i>		
BANN34	<i>Clostridium difficile</i>		
BANN35	<i>Clostridium sardiniense</i>		
BANN36	<i>Clostridium perfringens</i>		

^a pBCXO1 is pXO1-like, but not identical.

^b capA, B, and C are contained within the pXO2 plasmid of *Bacillus anthracis*; however, only the capA, B, and C sequences are found in 03BB102 and 03BB108.

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Note: In the case of a positive result, retest that strain 96 times with no failures allowed to demonstrate a 95% upper confidence limit on the POD of 0.05 or lower.

Environmental Interference

1 Definition

Ability of the assay to detect target organism in the presence of environmental substances and to be free of cross-reaction from environmental substances (*Annex A*).

2 Test Conditions

Test powders as liquid suspensions or solutions in the presence and absence of *Bacillus anthracis* Ames spores at the AMDL. Test swab materials in the presence and absence of *Bacillus anthracis* Ames spores at the AMDL.

3 Acceptance Criteria

No false positives and no false negatives observed.

Note: In the case of a false-positive or false-negative result, retest the material 96 times with no failures allowed.

Collaborative Validation Study

Reproducibility

1 Definition

Precision under conditions where independent test results are obtained with the same methods on equivalent test items in different laboratories with different operators using separate instruments.

2 Test Conditions

Test *Bacillus anthracis* Ames spores at AMDL and near neighbor organism at 10 times AMDL. At least 12 replicates per material per collaborator with 12 collaborators (four collaborators at each of three test sites).

3 Acceptance Criteria

Must produce at least 10 valid data sets. Report standard deviation of reproducibility (s_R).

POD at the AMDL Under Reproducibility Conditions (formerly termed System False-Negative Rate)

1 Definition

Rate of positive system results in a population of known positive test portions.

2 Test Conditions

Test *Bacillus anthracis* Ames spores at AMDL. At least 12 replicates per collaborator with 12 collaborators (four collaborators at each of three test sites).

3 Acceptance Criteria

Data for target agent must demonstrate an estimated 5% lower confidence limit on the CPOD of 0.95 or higher, where CPOD is the probability of detection calculated from pooled valid collaborative data.

POD in the Absence of Analyte Under Reproducibility Conditions (formerly termed System False-Positive Rate)

1 Definition

Rate of positive system results in a population of known negative test portions.

2 Test Conditions

Test near neighbor organism at 10 times AMDL. At least 12 replicates per collaborator with 12 collaborators (four collaborators at each of three test sites).

3 Acceptance Criteria

Data for near neighbor must demonstrate a 95% upper confidence limit on the CPOD of 0.05 or lower, where CPOD is the probability of detection calculated from pooled valid collaborative data.

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ANNEX A

Environmental Factors Panel for Validating HHAs for Biothreat Agents

1 Powders and Chemicals

Bacillus thuringiensis powders (e.g., Dipel)
Powdered milk
Powdered infant formula (Fe fortified)
Powdered infant formula (low Fe formulation)
Powdered coffee creamer
Powdered sugar
Talcum powder
Wheat flour
Baking soda
Chalk dust
Brewer's yeast
Dry wall dust
Cornstarch
Baking powder
GABA (Gama aminobutyric acid)
L-Glutamic acid
Kaolin
Chitin
Chitosan
MgSO₄
Boric acid
Powdered toothpaste
Popcorn salt

2 Swab Materials

Cotton swab with plastic shaft
Rayon swab with plastic shaft
Macrofoam swab with plastic shaft
Method Developer sample collection device

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