AOAC SMPR 2016.XXX; Version 4

Method Name: **Detection of Botulinum Neurotoxins A1 and A2**

Approval Body: *AOAC Stakeholder Panel on Agent Detection Assays*

1. **Intended Use**: Laboratory or field use by trained operators within the Department of Defense.

2. **Applicability**: Detection of Botulinum neurotoxins A1 and A2 in liquid samples. The preferential method would be a field-deployable assay or assays.

3. **Analytical Technique**: Any analytical method that can detect the protein and meets the requirements of this SMPR.

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 at a specified probability of detection (POD).

Maximum Time-To-Assay Result
Maximum time to complete an analysis starting with recovery of toxins from the collection matrix s and ending with the 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.

Selectivity Study
A study designed to demonstrate a candidate method's ability to detect the various forms of botulium neurotoxin A, and at the same time, demonstrate that a candidate method does not detect nontarget compounds and related nontarget toxins.

5. **System suitability tests and/or analytical quality control:**
The controls listed in Table I shall be made available in assays as appropriate. Manufacturer or method developer must provide written justification if controls are not available 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).

- Equal numbers of botulinum neurotoxin A1 and A2 and botulinum neurotoxin A1 and A2 complex samples must be represented in the selectivity study. Use pristine buffer

48 solution. Samples with target and nontarget compounds must be: 1) blind coded; 2)
49 randomly mixed together; 3) evaluated at the same time, and 4) masked, so that the
50 sample identity remains unknown to the analysts. Batches are permissible provided 6.1,
51 6.2, 6.3, and 6.4 are followed.
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53 • Information on other subtypes is desirable but not required.
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56 **7. Method Performance Requirements**
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Parameter	Minimum Performance Requirement
AMDL	1.25 ng /mL recovered Botulinum neurotoxin A1 and A2 complexes in liquid
Selectivity Study	POD ≥ 0.95 at AMDL for Botulinum neurotoxin A1 and A2 complex
	Tetanus toxin must test negative at 10x the AMDL†
System False-Negative Rate using spiked aerosol environmental matrix at the AMDL	≤ 5% (Annex I, Part 1)
System False-Positive Rate using aerosol environmental matrix at the AMDL	≤ 5% (Annex I, Part 1)
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[1]. 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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59 **8. Maximum Time for Assay Results:** Four hours
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[1] 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.

61 **Table 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 some method is utilized 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 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 (or sample set) run

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Annex I: 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. There are three parts to Environmental Factors studies: part 1 - environmental matrix samples; part 2 - the environmental organisms study; and part 3 - the potential interferants applicable to Department of Defense applications.[2] Part 2 is not applicable to techniques that do not detect nucleic acid; and therefore not included in this SMPR.

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.

[2] Added in June 2015 for the Deprtment of Defense project.

Part 2: Environmental Panel Organisms -

Not applicable to this SMPR and therefore removed.

Part 3: Potential Interferants Study

The Potential Interferants Study supplements the Environmental Factors Study, and is applicable to all biological threat agent detection assays for Department of Defense applications. Table V provides a list of potential interferants that are likely to be encountered in various Department of Defense applications.

Method developers and evaluators shall determine the most appropriate potential interferants for their application. Interferants shall be spiked at a final test concentration of 1 µg/ml directly into the sample collection buffer. Interferants may be pooled. Sample collection buffers spiked with potential interferants 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.

It is expected that all samples are correctly identified as positive. If using pooled samples of potential interferants, and a negative result occurs, then the pooled potential interferants shall be tested separately at the 2 times the AMDL (or AMIL) with one of the target biological threat agents.

Table 1A: Potential Interferants

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

Table 4 is offered for guidance and there are no mandatory minimum requirements for the number of potential interferants to be tested.

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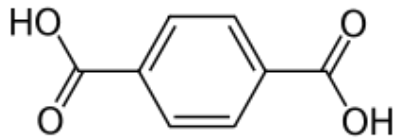
[1] **JP-8**. Airforce formulation jet fuel.

[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.

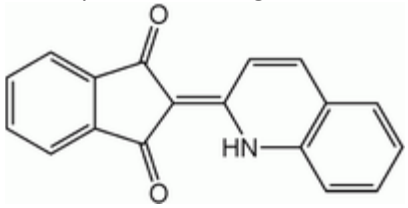
[3] **Burning rubber** (tire smoke). Gaseous C1-C5 hydrocarbons: methane; ethane; isopropene; butadiene; propane. Polycyclic aromatic hydrocarbons (58-6800 ng/m^3): parabenzo(a)pyrene; polychlorinated dibenzo-p-dioxins (PCDD); polychlorinated dibenzofurans (PCDF). Metals (0.7 - 8 mg/m^3): zinc; lead;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