Appendix O: Environmental Factors for Validating Biological Threat Agent Detection Assays

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 Organisms; Part 2—Environmental Matrix Samples;* and *Part 3—Potential Interferents* applicable to Department of Defense applications (added in June 2015 for the Department of Defense project).

Part 1: Environmental Organisms

1.1 Bioinformatics Analyses

In silico screening can be performed on all nucleic acid signature sequences used in assays (e.g., primers, probes, amplicons, etc.) to demonstrate specificity to the target biological threat agent.

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 organism/strain be included in the exclusivity or inclusivity panels, respectively, if the strains are available.

A method developer-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, databases, and/or published documents describing the genetic sequences found in soils that are representative of the regions of operation. 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 into predictions. The program should detect possible amplicons from any selected database of sequences.

Potential tools for *in silico* screening of nucleic acid sequences 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 sequences.

- NCBI tools

The method developer 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 the tool(s) used for bioinformatics evaluation: Data demonstrating the selected tool(s) 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 organisms and/or strains to be added to the inclusivity or exclusivity panels based on the bioinformatics evaluation.

1.2 Environmental Organisms

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.

If bioinformatic analysis is completed, then DNA from Group 1 organisms should be tested. If bioinformatic analysis is not completed, then DNA from Groups 1 and 2 organisms should be tested.

Group 1 organisms should be tested individually at a DNA concentration equivalent to 10x acceptable minimum detection level (AMDL). Group 2 organisms may be tested as pools containing 10 organisms each represented at 10x the AMDL. These tests should be negative for the target sequence. The same pools can be spiked with 2x the AMDL for the target organism and tested. These tests should be positive for the target in the high DNA background. If an unexpected result occurs, each of the individual environmental organisms from a failed pool must be individually retested at 10x the AMDL with and without the target gene or gene fragment at 2x the AMDL in the candidate method DNA elution buffer.

DNA from organisms on this list that already appears in the inclusivity or exclusivity panel does not need to be tested again as part of the environmental factors panel.

Group 1: Eukaryotic Organisms Possibly Present in Air, Soil, and Water

- *Aedes aegypti* (ATCC® CCL-125™ 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, USA)
- Gypsy moth cell lines LED652Y cell line (baculovirus; Invitrogen)

- Cockroach (commercial source)
- Tick (*Amblyomma* and *Dermacentor* tick species for *F. tularensis* detection assays)
- *Mus musculus* (ATCC/HB-123) mouse
- *Rattus norvegicus* (ATCC/CRL-1896) rat
- *Homo sapiens* (HeLa cell line ATCC/CCL-2) human

Group 2: Cultivatable Bacteria Possibly 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 gladioli*
- *Burkholderia stabilis*
- *Burkholderia plantarii*
- *Chryseobacterium indologenes*
- *Clostridium sardiniense*
- *Clostridium perfringens*
- *Deinococcus radiodurans*
- *Delftia acidovorans*
- *Escherichia coli* K12
- *Fusobacterium nucleatum*
- *Lactobacillus plantarum*
- *Legionella pneumophila*
- *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*

Part 2: Environmental Matrix Samples

2.1 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.

Aerosol samples should be spiked at 0x, 2x, 5x, and 10x AMDL with the archetype organism [usually specified in the *Standard Method Performance Requirements* (SMPRs®) for AMDL testing, such as strain CO92 for *Yersinia pestis*] and then evaluated for inhibition and cross reactivity. At 0x a negative reaction is expected, and at 2x, 5x, and 10x a positive reaction is expected. Testing should be done using intact target organisms so that potential problems with the DNA extraction can be determined.

2.2 Soil Testing

Airborne soil particles may constitute a significant challenge to the analysis of collected aerosol samples. Soils contain genomic materials or nucleic acid fragments of countless archaebacterial, bacterial, and eukaryotic organisms. Some of the more common soil organisms can be anticipated. Soils may also contain unanticipated components that interfere with extraction, denaturation, polymerization, or annealing reactions. Therefore, determining the effect of a variety of representative soils on the robustness of a PCR assay is an important first step.

Justifications for the selected soils that were used must be documented.

Samples of each regional soil type [Arizona Test Dust is available as a baseline starting point. *See* Section 1.1 *Bioinformatics Analysis* on probing all available databases, including those that contain soil metagenome sequences generated from specific regions of operations (if available) for *in silico* analysis and further validation of the signature sequences.] should be spiked at 0x, 2x, 5x, and 10x AMDL with the archetype organism (usually specified in the SMPR for AMDL testing, such as strain CO92 for *Yersinia pestis*) and then evaluated for inhibition and cross reactivity. At 0x a negative reaction is expected, and at 2x, 5x, and 10x a positive reaction is expected. Testing should be done using intact target organisms so that potential problems with the DNA extraction can be determined.

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 1 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. Interferents may be pooled. Sample collection buffers spiked with potential interferents shall by inoculated at 2x the AMDL [or acceptable minimum identification level (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 µg/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. If using pooled samples of potential interferents, and a negative result occurs, then the pooled potential interferents shall be tested *separately* at 2x the AMDL (or AMIL) with one of the target biological threat agents.

Table 1. Potential interferents

Compound		Potential theaters of operation
Group 1: Petroleum-based	JP-8[a]	Airfield
	JP-5[b]	Naval
	Diesel/gasoline mixture	Ground
	Fog oil (standard grade fuel No. 2)	Naval, ground
	Burning rubber[c]	Ground, airfield
Group 2: Exhaust	Gasoline exhaust	Ground
	Jet exhaust	Naval, airfield
	Diesel exhaust	Ground
Group 3: Obscurants	Terephthalic acid[d]	Ground
	Zinc chloride smoke[e]	Ground
	Solvent yellow 33[f]	Ground
Group 4: Environmental	Burning vegetation	Ground, airfield
	Road dust	Ground
	Sea water (sea spray)	Naval
Group 5: Chemicals	Brake fluid[g]	All
	Brake dust[h]	Ground
	Cleaning solvent, MIL-L-63460[i]	All
	Explosive residues: High explosives[j] Artillery propellant[k]	All

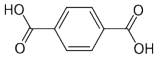
Note: Table 1 is offered for guidance, and there are no mandatory minimum requirements for the number of potential interferents to be tested.

[a] JP-8. Air Force formulation jet fuel.

[b] 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.

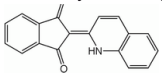
[c] Burning rubber (tire smoke). Gaseous C1-C5 hydrocarbons: methane, ethane, isopropene, butadiene, propane. Polycyclic aromatic hydrocarbons (58–6800 ng/m^3): parabenzo(*a*)pyrene, polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF). Metals (0.7–8 mg/m^3): zinc, lead, cadmium.

[d] Terephthalic acid. Used in the AN/M83 hand grenade currently used by U.S. military.



[e] Zinc chloride smoke. Also known as “HC smoke.” Was used in the M8 grenade and still used in 155 mm artillery shells. HC smoke is composed of 45% hexachloroethane, 45% zinc oxide, and 10% aluminum.

[f] Solvent yellow 33 [IUPAC name: 2-(2-quinolyl)-1,3-indandione] is a new formulation being develop for the M18 grenade.



[g] 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 and requires less maintenance.

[h] 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.

[i] MIL-L-63460, “Military Specification, Lubricant, Cleaner and Preservative for Weapons and Weapons Systems;” trade name “Break-Free CLP.”

[j] High explosives. The M795 155 mm projectile is the U.S. Army/Marine Corp’s current standard projectile containing 10.8 kg 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.

[k] 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.