MICROBIOLOGICAL METHODS

RAZOR® EX Anthrax Air Detection System

***Performance Tested Method*SM 101103**

Abstract

The RAZOR® EX Anthrax Air Detection System, developed by Idaho Technology, Inc. (ITI), is a qualitative method for the detection of *Bacillus anthracis* spores collected by air collection devices. This system comprises a DNA extraction kit, a freeze-dried PCR reagent pouch, and the RAZOR EX real-time PCR instrument. Each pouch contains three assays, which distinguish potentially virulent *B. anthracis* from avirulent *B. anthracis* and other *Bacillus* species. These assays target the pXO1 and pXO2 plasmids and chromosomal DNA. When all targets are detected, the instrument makes an "anthrax detected" call, meaning that virulence genes of the anthrax bacillus are present. This report describes results from AOAC Method Developer (MD) and Independent Laboratory Validation (ILV) studies, which include matrix, inclusivity/exclusivity, environmental interference, upper and lower LOD of DNA, robustness, product consistency and stability, and instrument variation testing. In the MD studies, the system met the acceptance criteria for sensitivity and specificity, and the performance was consistent, stable, and robust for all components of the system. For the matrix study, the acceptance criteria of 95/96 expected calls was met for three of four matrixes, clean dry filters being the exception. Ninety-four of the 96 clean dry filter samples tested gave the expected calls. The nucleic acid limit of detection was 5-fold lower than AOAC's acceptable minimum detection limit. The system demonstrated no tendency for false positives when tested with *Bacillus cereus*. Environmental substances did not inhibit accurate detection of *B. anthracis*. The ILV studies yielded similar results for the matrix and inclusivity/exclusivity studies. The ILV environmental interference study included environmental substances and environmental organisms. Subsoil at a high concentration was found to negatively interfere with the pXO1 reaction. No interference was observed from the environmental organisms. The nucleic acid LOD, however, was 10 times higher (1 pg/reaction, equivalent to about 200 spores) than that found in the MD study. These results indicate that the RAZOR System is a sensitive and specific system that accurately identifies *B. anthracis* in aerosol matrixes and in the presence of interfering substances, and that the method can be performed by an independent laboratory and achieve similar results.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*SM. *See* http://www.aoac.org/testkits/steps.html for information on certification.

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Scope of Method

The RAZOR EX Anthrax Air Detection System (referred to henceforth as the RAZOR Anthrax System) is designed to determine the presence or absence of *B. anthracis* in aerosol collection samples. It should be used in an appropriate biological laboratory following Centers for Disease Control and Prevention (CDC) guidelines outlined in the *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), 2009, CDC, and National Institutes of Health (NIH) by laboratory personnel trained in the use of the method and trained in BSL-2/3 practices.

Definitions

Acceptable minimum detection level (AMDL) is defined as the predetermined minimum level of a biological threat agent, as specified by the AOAC Methods Committee on Biological Threat Agents with input from subject matter experts, that must be detected by the candidate method. The estimated 5% lower confidence limit on the probability of detection (POD) must be 0.95 or higher. The AMDL is dependent on the intended use. For *B. anthracis* PCR methods, the AMDL for studies using live spores is 20 000 standardized *B. anthracis* Ames spores/filter, or 2000 standardized *B. anthracis* Ames spores/mL for liquid collection methods. For studies using purified nucleic acid, the AMDL is 2000 genome equivalents (GE)/mL (11 pg DNA/mL). The monoploid chromosome number of *B. anthracis* is one (1). Although two copies of the chromosome have been reported for its neighbors *B. cereus, B. megaterium,* and *B. thuringiensis* (2), this has not been established in *B. anthracis* strains, despite multiple complete genome based investigations (1, 3). The 2000 GE/mL set as AMDL for studies using purified nucleic acid was intended to represent 2000 organisms/mL.

Inclusivity is defined as the strains, isolates, or variants of the target agent(s) that the method can detect.

Environmental interference is defined as either cross reactivity or inhibition due to contaminants likely to be encountered under intended use conditions.

Exclusivity is defined as the nontarget agents, which are potentially cross-reactive, that are not detected by the method.

Standardized dust is defined as a mixture of dusts collected from multiple states at various locations within each state. The dusts were processed to a particle size of 40 microns. These dusts were verified to be negative for the target genes by PCR testing. Dusts were mixed to homogeneity and stored as 50 g aliquots under vacuum.

POD is defined as 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.

Product consistency is defined as the consistency of the product performance across manufacturing lots.

Product stability is defined as the consistency of product performance over time, under normal storage conditions for the claimed shelf-life of the product.

Robustness is defined as the capacity of a method to remain unaffected by small but deliberate variations in method parameters and is an indication of its reliability during normal usage.

The AOAC Stakeholder Panel on Agent Detection Assays (SPADA) is a balanced group of stakeholders and subject matter experts convened to develop standard method performance requirements for biothreat detection methods for various intended uses.

Principle

The RAZOR Anthrax System is a real-time PCR method in a pouch format using the RAZOR EX instrument. If target DNA is present during PCR, a fragment of the target DNA is amplified using specific primers. The amplified target is detected by fluorescence using a specific pair of hybridization probes with fluorophores (4). These probes consist of two different short oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the reaction cycle. After hybridization to the template DNA, the two probes are in close enough proximity to permit fluorescence resonance energy transfer (FRET) between the two fluorophores.

General Information

B. anthracis, a Gram-positive, spore-forming bacterium, is the causative agent of anthrax. Humans can become infected with *B. anthracis* through inhalation, ingestion, or cutaneous exposure. Because of the stability of the spores, the organism can be an effective biological weapon, especially when dispersed by aerosol release. Inhalation exposure causes respiratory distress followed shortly by shock and death. The mortality rate of inhalation anthrax is close to 100% in the later stages despite aggressive treatment (5).

B. anthracis contains two plasmids required for virulence: pXO1, which encodes the toxins and toxin delivery protein; and pXO2, which encodes the capsule (6, 7). Target sequences on each of the plasmids, coupled with a target sequence on the chromosomal DNA, are the basis for the specificity of the RAZOR Anthrax System.

Test Kit Information

(**a**) *Test kit name.*—RAZOR EX Anthrax Air Detection System.

(**b**) *Cat. No.*—PATH–ASY–0095.

(**c**) *Ordering information.*—United States: 800-735-6544, Europe: +1-801-736-6354.

(**d**) *Test kit components.*—

Component 1.—Pouch containing freeze-dried reagents in a foil bag.

Component 2.—Antifoam powder.

Component 3.—Reagent grade water.

Component 4.—Sample buffer.

Component 5.—Medium bead tube with beads.
Component 6.—Control buffer.
Component 7.—Fine-tipped transfer pipet.
Component 8.—MagBead strip tubes.
Component 9.—1 mL syringes with cannulae attached.
Component 10.—Protocol instruction booklet.

Additional Supplies

(**a**) *Pipettors*.—20–1000 µL range.
(**b**) *Pipet tips*.—With aerosol filters.
(**c**) *Peg racks*.—VWR, www.vwr.com, No. 82024-496; one rack required per eight samples.
(**d**) *Biocontrol PickPen® 1 M magnetic wand*.—Sunrise Science, www.sunrisescience.com, No. 23001.
(**e**) *Biocontrol PickPen tip box*.—Sunrise Science, No. 34196.
(**f**) *Biocontrol PickPen tips*.—Sunrise Science, No. 34500.
(**g**) *Phosphate buffered saline (PBS) packets*.—Sigma, www.sigmaaldrich.com, No. P3813-10PAK.
(**h**) *Triton® X-100*.—Sigma, No. X100-100 mL.

Apparatus

(**a**) *RAZOR EX system*.—Idaho Technology, Inc. (ITI) RAZR-RED-4000, with RAZOR EX operator's manual.
(**b**) *Disruptor Genie™*.—Scientific Industries, www.scientificindustries.com, No. SI-D237 (or Vortex Genie® 2T with Turbomix™ attachment, No. SI-T236) and adapter for 2 mL tubes (No. SI-0562).

Standard Reference Materials (SRM)

(**a**) *B. anthracis strain Canadian Bison*.—Midwest Research Institute (MRI, 1470 Treeland Blvd SE, Palm Bay, FL 32909), No. 107448.
(**b**) *B. anthracis strain V770-NP-1R*.—MRI, No. 107240.
(**c**) *B. anthracis strain PAK-1*.—MRI, No. 107518.
(**d**) *B. anthracis strain BA1015*.—MRI, No. 107446.
(**e**) *B. anthracis strain Ames*.—MRI, No. 107517.
(**f**) *B. anthracis strain K3*.—MRI, No. 107497.
(**g**) *B. anthracis strain Ohio ACB*.—MRI, No. 107339.
(**h**) *B. anthracis strain SK-102*.—MRI, No. 107449.
(**i**) *B. anthracis strain Vollum 1B*.—MRI, No. 107539.
(**j**) *B. anthracis strain BA1035*.—MRI, No. 107451.
(**k**) *B. anthracis strain RA3*.—MRI, No. 107520.
(**l**) *B. anthracis strain 2002013094 (240)*.—MRI, No. 124030.
(**m**) *B. anthracis strain Pasteur*.—MRI, No. 107171.
(**n**) *B. anthracis strain Sterne*.—MRI, No. 107453.
(**o**) *B. anthracis strain Turkey No. 32*.—MRI, No. 107255.
(**p**) *B. cereus strain S2-8*.—MRI, No. 122840.
(**q**) *B. cereus strain 3A*.—MRI, No. 122841.
(**r**) *B. thuringiensis strain HD1011*.—MRI, No. 122842.
(**s**) *B. thuringiensis strain 97-27*.—MRI, No. 122843.
(**t**) *B. thuringiensis strain HD682*.—MRI, No. 122844.
(**u**) *B. cereus strain E33L*.—MRI, No. 122845.
(**v**) *B. cereus strain D17*.—MRI, No. 122846.
(**w**) *B. thuringiensis strain HD571*.—MRI, No. 122847.
(**x**) *B. cereus strain Al Hakam*.—MRI, No. 122848.
(**y**) *B. cereus strain ATCC 4342*.—MRI, No. 122849.
(**z**) *B. cereus strain FM1*.—MRI, No. 122850.
(**aa**) *B. cereus strain G9241*.—MRI, No. 122851.
(**bb**) *B. cereus strain 03BB102*.—MRI, No. 122852.
(**cc**) *B. cereus strain 03BB108*.—MRI, No. 122853.
(**dd**) *B. thuringiensis subsp. Israelensis*.—MRI, No. 122857.
(**ee**) *B. thuringiensis subsp. Kurstaki*.—MRI, No. 122858.
(**ff**) *B. thuringiensis subsp. Morrisoni*.—MRI, No. 122859.
(**gg**) *B. coagulans strain ATCC 7050*.—MRI, No. 122854.
(**hh**) *B. mycoides strain ATCC 6462*.—MRI, No. 122855.
(**ii**) *B. megaterium strain ATCC 12872*.—MRI, No. 122856.
(**jj**) *Loam*.—SRM:SLM071709.xx (where xx denotes the bottle number of the batch).
(**kk**) *Sandy loam*.—SRM:SSL071609.xx (where xx denotes the bottle number of the batch).
(**ll**) *Clay*.—SRM:SC071709.xx (where xx denotes the bottle number of the batch).
(**mm**) *Subsoil*.—SRM:SSS071609.xx (where xx denotes the bottle number of the batch).
(**nn**) *Silt*.—SRM:SLT071609.xx (where xx denotes the bottle number of the batch).
(**oo**) *Dipel (B. thuringiensis powder)*.—MRI, No. P.060909BT.1.1.
(**pp**) *Powdered milk*.—MRI, No. P.060509MK.1.
(**qq**) *Powdered infant formula, high Fe*.—MRI, No. P.073109FH.
(**rr**) *Powdered infant formula, low Fe*.—MRI, No. P.073109FL.1.
(**ss**) *Powdered coffee creamer*.—MRI, No. P.080509CC.1.
(**tt**) *Powdered sugar*.—MRI, No. P.060509SG.1.
(**uu**) *Talcum powder*.—MRI, No. P.060509.TP.1.
(**vv**) *Wheat flour*.—MRI, No. P.060509.WF.1.
(**ww**) *Baking soda*.—MRI, No. P.060509BS.1.
(**xx**) *Chalk dust*.—MRI, No. P.060909CK.1.
(**yy**) *Brewer's yeast*.—MRI, No. P.060909YT.1.
(**zz**) *Dry wall dust*.—MRI, No. P.060809.DW.1.
(**aaa**) *Corn starch*.—MRI, No. P.073109CS.1.
(**bbb**) *Baking powder*.—MRI, No. P.073109BP.1.
(**ccc**) *γ-Aminobutyric acid*.—MRI, No. P.080409GB.1.
(**ddd**) *L-Glutamic acid*.—MRI, No. P.080409.GA.1.
(**eee**) *Kaolin*.—MRI, No. P.080509KN.1.
(**fff**) *Chitin*.—MRI, No. P.080509CN.1.
(**ggg**) *Chitosan*.—MRI, No. P080409CT.1.
(**hhh**) $MgSO_4$.—MRI, No. P.080509MG.1.
(**iii**) *Boric acid*.—MRI, No. P.081209BA.1.
(**jjj**) *Powdered toothpaste*.—MRI, No. P.081009.TO.
(**kkk**) *Popcorn salt*.—MRI, No. P.073109PS.1.
(**lll**) *Standardized dust*.—SRM:012308.xx (where xx denotes the bottle number of the batch).

Standard Solutions

(**a**) *PBS*.—Prepare 1 L from PBS packets according to package instructions.
(**b**) *PBS with 0.1% Triton X-100*.—Add 1 mL Triton X-100 to 1 L PBS.

Safety Precautions

To avoid contamination, filter tips should be used on pipets when working with any liquid solution.

A Type II biosafety cabinet must be used for any procedures where there is a possibility of creating aerosols or splashes.

When working with potentially infectious samples or chemicals, the appropriate personal protective equipment must be worn, including laboratory coat, latex or nitrile gloves, and eye protection.

Avoid exposure to any potentially infectious samples or harmful chemicals. Exposure can occur by inhalation, ingestion, or skin absorption.

For more information on kit components, consult the appropriate material safety data sheet (MSDS) provided by ITI.

For general biosafety guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories* (5th Ed.), February 2009, CDC and NIST; available online at http://www.cdc.gov/biosafety/publications/bmbl5/index.htm.

Use universal precautions when handling all infectious or potentially infectious samples. Dispose of used reagent vials in accordance with local and state regulations. Before disposal, waste from possible biohazardous samples should be inactivated using appropriate procedures and in accordance with local and state regulations.

General Preparation

Before beginning work, decontaminate all work surfaces and equipment with a paper towel moistened with 10% household bleach followed by a paper towel moistened with distilled water.

Allow frozen samples to thaw.

Assemble all materials necessary for testing, including reagents, pouches, and forms.

If the RAZOR EX is to be run on a battery, ensure that it has sufficient charge to allow the test to be completed. A fully charged battery allows seven to eight pouches to be tested. Follow directions in the Protocol Instruction Booklet that accompanies the kit.

Sample Preparation

For air collection filters, DFU-P-24 (Lockheed Martin, Gaithersburg, MD; 47 mm), filter was used for validation:

(*1*) Place dry filter in a 50 mL conical tube with collection side facing away from the side of the tube.

(*2*) Add 7 mL PBS with 0.1% Triton X-100 to the conical tube.

(*3*) Cap tube and shake by hand for 2 min.

(*4*) Proceed to *Preparation and Lysis*.

For air collection liquids:

(*1*) Liquid must be PBS or PBS with 0.1% Triton X-100.

(*2*) Proceed to *Preparation and Lysis*.

Preparation and Lysis:

(*1*) Add powdered antifoam to medium bead tube by scraping a small amount of antifoam from the applicator onto the inside lip of the tube. Tap tube on a hard surface to let the antifoam settle to the bottom of the tube.

(*2*) Use pipettor to add 1.2 mL liquid sample to medium bead tube containing antifoam.

(*3*) Cap tube tightly.

(*4*) Bead beat for 5 min in Disruptor Genie by placing bead tube in 2.0 mL tube adaptor with cover in the down position.

Bind nucleic acids to magnetic beads:

(*1*) Open MagBead strip tube by peeling back cover. The first well in the MagBead strip tube has magnetic beads that are black in color.

(*2*) Use a small transfer pipet to transfer sample (supernatant) from the bead tube to the first well in the MagBead strip tube. Be careful to avoid transferring the white beads.

(*3*) Mix sample with magnetic beads by pipetting up and down with small transfer pipet.

(*4*) Binding incubation: Incubate at room temperature for at least 8 min (a longer incubation is acceptable, up to 45 min).

(*5*) Place PickPen tip on the PickPen wand.

(*6*) To lock magnet on, slide switch (found on the side of the PickPen wand) down and over in slot until magnetic tip is fully extended and locked into place. The magnet must be locked into place to properly collect the magnetic beads.

(*7*) To unlock the magnet, slide switch over and up in slot until magnetic tip is retracted.

Notes: The same PickPen tip is used for washing and eluting. Well 1 is on the side of the elongated tab strip tube.

(*8*) To collect the magnetic beads from the first (binding) well, lock magnet on and move the PickPen wand with a circular and up and down motion in the solution for about 30–45 s.

(*9*) Transfer the magnetic beads into the second well by unlocking the magnet and gently moving wand around in the solution until the magnetic beads come off the PickPen tip into the wash buffer.

(*10*) Transfer more magnetic beads from the first well to the second by repeating steps 8 and 9.

(*11*) To collect the magnetic beads from the second (wash) well, lock magnet on and move the PickPen wand with a circular and up and down motion in the solution for about 5–10 s.

(*12*) Transfer the magnetic beads into the third well and gently release them by unlocking the magnet and gently moving the wand around in the solution until they come off the PickPen tip into the wash buffer.

(*13*) To collect the magnetic beads from the third (wash) well, lock magnet on and move the PickPen wand with a circular and up and down motion in the solution for about 5–10 s.

(*14*) Transfer the magnetic beads into the fourth well and gently release them by unlocking the magnet and gently moving the wand around in the solution until the magnetic beads come off the PickPen tip into the wash buffer.

(*15*) To collect the magnetic beads from the fourth (wash) well, lock magnet on and move the PickPen wand with a circular and up and down motion in the solution for about 5–10 s.

(*16*) Transfer the magnetic beads into the last well and release them by unlocking the magnet. Vigorously move wand around in the elution buffer until all of the magnetic beads have come off the tip. Use the end of the tip to break up any magnetic bead clumps in the elution buffer.

(*17*) Incubate magnetic beads in the elution buffer at room temperature for at least 2 min.

(*18*) To remove magnetic beads, lock magnet on and move the PickPen wand with a circular and up and down motion in the solution for about 5–10 s. Discard beads with tip by using tip ejector button (top of PickPen). This yields approximately 200 µL of eluate.

(*19*) Transfer purified sample to a RAZOR sample buffer bottle with a pipettor or a small transfer pipet.

Analysis

Reagent pouch preparation.—The RAZOR EX instrument is equipped with an onboard screen that walks the user through each step of preparing and loading a pouch into the instrument.

Before performing a run, the user is prompted to load the pouch run protocol. Run protocols are loaded onto the instrument by scanning the square bar code found on the reagent box. Scanning the rectangular bar code on the RAZOR reagent pouch indicates which protocol is to be used for running the pouch. For complete instructions, refer to "loading protocols" in Chapter 2, Installation and Setup, of the RAZOR EX operator's manual.

(*1*) After the square bar code has been loaded, select Run Pouch to scan the rectangular bar code. The Remove Old Pouch screen will be displayed. To remove the pouch, pull it out of the pouch slot.

(*2*) Press Select. The Inject All Ports screen will be displayed.

(*3*) Remove the freeze-dried reagent pouch from the aluminum can and air-tight foil bag.

Caution: Do not remove the pouch from its packaging unless that pouch can be tested within 30 min.

(*4*) Place the pouch on a flat, clean surface with the inlet ports and label face up. Make sure the plunger comb is in place.

(*5*) Load the pouch in the following order to minimize cross contamination and user error:

(*i*) *Negative port*.—Add 0.5 mL control buffer.

(*ii*) *Unknown 1 port*.—Add 0.5 mL sample 1 in sample buffer.

(*iii*) *Unknown 2 port*.—Add 0.5 mL sample 2 in sample buffer.

(*iv*) *Positive port*.—Add 0.5 mL control buffer.

(*6*) Uncap the cannula on the syringe. The end of the syringe is preloaded with a cannula tip necessary for pouch loading. Twist the cannula to confirm that it is tightly attached to the end of the syringe.

(*7*) To avoid splashing, slowly insert the cannula end of the syringe into the control buffer or sample.

Caution: If there are air bubbles in the syringe, DO NOT remove them by tapping and then pushing bubbles out of the cannula tip into the air (this could contaminate both you and your area). Instead, reinsert the syringe into the sample vial and slowly express the liquid. Slowly redraw the sample into the syringe. Repeat until the liquid in the syringe is free of air bubbles.

(*8*) Draw the 0.5 mL control buffer or sample into the syringe by pulling up on the syringe plunger. Avoid introducing any air into the system, which can cause bubbles in the pouch.

(*9*) Hold the syringe by the syringe body and gently insert the cannula tip into the correct inlet port. Push the syringe down until you feel a faint pop and an ease in resistance. A broken inlet port allows the liquid to be pulled into the pouch by vacuum. A single port hydrates three slots (100 µL /slot), each slot representing a different target assay. The syringe should sit in the inlet port for at least 30 s so the liquid can disperse evenly. Once the syringe plunger has stopped moving, remove the syringe and discard it in a biowaste container. Repeat for each inlet port.

Caution: Do not push the syringe plunger to force liquid into the pouch. This can fill the pouch with air and may damage it or cause contamination.

Note: If the liquid does not flow into the pouch automatically, refer to the pouch troubleshooting section or use a new reagent pouch.

Note: Return any remaining sample to the sample tube in case retesting is required.

Loading the Pouch into the RAZOR EX:

Note: Be sure that the outside of the pouch looks clean before you insert it into the instrument. If the pouch looks unclean, gently wipe it with a dry, lint-free cloth.

(*1*) Press Select on the RAZOR EX. The Preheating screen will display followed by Preheat Complete.

(*2*) Press Select again to display Remove Comb.

(*3*) Remove all the plunger combs by pulling them away from the pouch.

(*4*) Press Select to display Twist Plungers.

(*5*) Using the plunger twist tool, rotate each of the plungers 90° toward center of pouch; this prevents leaking and cross-contamination of neighboring wells.

(*6*) With the pouch plungers up, lightly tap the pouch on a flat surface.

(*7*) Press Select to display Use Tool to Plunge.

Note: DO NOT push pouch plungers down until "Use Tool to Plunge" stage or until prompted on the RAZOR EX screen.

Caution: Pressing the pouch plungers when the pouch is not in the pouch bracket may damage it, prevent the sample from entering the sample slot, or cause backflow leading to contamination.

(*8*) Place the pouch onto the plunging tool located near the pouch slot on the instrument.

(*9*) Hold the reagent pouch in the twist tool bracket and slowly push the plungers down to force the liquid into the individual sample slots. When all of the plungers have forced liquid into the sample pouch, you are now ready to run the samples.

(*10*) Press Select. The Analysis Initiation screen will display. Select Proceed.

(*11*) The Insert Pouch screen will display, at which point you should insert the pouch into the pouch slot and press Select.

(*12*) The RAZOR EX instrument will begin the run, and a PCR Runtime Menu screen will display four view options from which the progress of the run can be monitored.

Interpretation and Test Results

Understanding the Pouch Slots.—The system uses three separate types of reagents.

Negative (–) control slots provide a check to verify that the reagent pouch was not contaminated. If the test runs correctly, these slots will be negative.

The Unknown (U) slots contain the unknown samples that are being tested.

Positive (+) control slots are designed to confirm that the instrument and reagents work. If the test runs correctly, these slots will be positive.

Table 1. Call symbols displayed on the instrument screen during the course of a run

Calls	Meaning of each call
+	Software has detected an amplification curve for a slot position.
–	Software has not detected an amplification curve for a slot position.
?	Software has not determined the correct call yet.

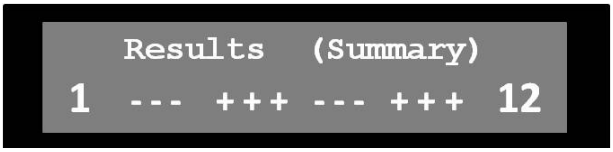

Figure 1. Results summary screen on a RAZOR EX instrument.

Detector.—When a run is finished, the screen automatically displays the results (Metacalls) for the run. These results rely on the Detector software to determine the presence or absence of the target being tested.

During the run, the instrument screen displays the status of amplification in each pouch slot (Table 1). Detector software uses an advanced mathematical algorithm that determines if each slot in the pouch has detectable amplification (that is, whether copies were made of the target DNA). Slots are analyzed independently of each other. Detector runs in real time on the instrument: strongly positive slots are called positive while the machine runs, and the Detector calls at the top of the screen are updated. Negatives are not called until the run is over. All samples will have a positive or negative call at the end of each run.

The Metacalls program uses the Detector calls for individual slots and provides combined information about unknowns and assay controls.

Run screen.—Each run screen displays the pouch number, the name of the protocol, and the date and time of the run, as well as the following options:

Results (summary).—Combined calls (Metacalls) for unknowns and controls.

Results (details).—Specific results for each pouch slot.

View graph.—A graph of the test results.

Results (summary).—Figure 1 is an example of what you will see when you look at a summary of results. The first line is the screen title. The second line starts with the number 1 on the left side of the line and ends with the number 12 on the right side of the line. The number 1 represents slot 1 and the number 12 represents slot 12. Slots 1–3 provide the results for the negative control, slots 4–6 and 7–9 provide the results for the unknown samples, and slots 10–12 provide results for the positive control. Minus (–) or plus (+) symbols are detector call symbols. Plus (+) symbols indicate that amplification was detected in the slot.

Results interpretation.—Table 2 explains how the combination of calls and possible retest are to be interpreted. If the sample is retested, the calls should be interpreted as explained in Table 3.

Method Developer (MD) Validation Studies

The AOAC Method Developer Validation Study Protocol, "PCR Methods for Detection of *Bacillus anthracis* Spores from Filters or Liquid Aerosol Collection Samples" (Protocol No. BA-01, Revision 2, Approval Date: 6/28/10), was followed for all MD validation studies. All samples were prepared by MRIGlobal's Palm Bay, FL (MRI-FL) facility. ITI conducted the DNA-based method developer studies [inclusivity/exclusivity, upper and lower LODs of DNA, and product consistency and stability of PCR reagent (RAZOR EX pouches)] in their Salt Lake City, UT facility under BSL-2 conditions. Studies involving live spores [matrix studies, environmental interference, robustness, product consistency and stability (DNA extraction kit), and instrument variation] were contracted to MRIGlobal's Kansas City, MO (MRI-KC) site and performed in the BSL-3 facility.

Inclusivity and Exclusivity

Fifteen strains of *B. anthracis* and 20 closely related organisms were used to evaluate the inclusivity and exclusivity of the RAZOR Anthrax System. The strains were selected by SPADA, and tested as purified DNA at the AMDL (2000 genome equivalents/mL, which is approximately 11 pg/mL) for inclusivity strains and at 10x AMDL (110 pg/mL) for exclusivity strains. The SPADA acceptance criteria are 100% expected results for inclusivity and exclusivity organisms.

Methodology.—Each strain was cultured in 200 mL brain heart infusion (BHI) or trypticase soy broth (TSB), depending on which better supported the growth of each organism, at 37°C with shaking (120 rpm) for 3.5 to 6 h. Each culture was divided into ten 20 mL aliquots and centrifuged to pellet the cells. The

Table 2. Initial test results interpretation chart

Call for each target					
pXO1 Ant T2	pXO2 Ant T3	Chromosome Ant T4	MetaCall	Interpretation	Action
+	+	+	Anthrax detected	Positive for pXO1, pXO2, and *B. anthracis* chromosome[a]	Test complete, report MetaCall
–	–	–	Anthrax not detected	Negative for *B. anthracis*	
+	+	–		pXO1 and pXO2 detected	
+	–	+		pXO1 and *B. anthracis* chromosome detected	
+	–	–	Incomplete detection	pXO1 detected	Retest
–	+	+		pXO2 and *B. anthracis* chromosome detected	
–	+	–		pXO2 detected	
–	–	+		*B. anthracis* chromosome detected	

[a] The test detects the presence of genes for virulence determinants. These results, when accompanied by a viable isolate, suggest the presence of virulent *B. anthracis*.

Table 3. Retest results interpretation chart

Retest call for each target					
pXO1 Ant T2	pXO2 Ant T3	Chromosome Ant T4	MetaCall	Retest result	Action
+	+	+	Anthrax detected	Positive for pXO1, pXO2, and *B. anthracis* chromosome[a]	Test complete, report MetaCall
–	–	–	Anthrax not detected	All targets negative	Re-prep sample and retest after decontamination
+	+	–	Incomplete detection[b]	(*1*) Same combination of positive and negative calls as first pouch	(*1*) Accept and report interpretation from first pouch[b]
+	–	+			
+	–	–			
–	+	+		(*2*) Combination of positive and negative calls different from first pouch	(*2*) Re-prep sample and retest
–	+	–			
–	–	+			

[a] The test detects the presence of genes for virulence determinants. These results, when accompanied by a viable isolate, suggest the presence of virulent *B. anthracis*.

[b] Incomplete detection may indicate the presence of a *Bacillus* species that does not contain all the target markers.

supernate was removed and the cell pellets stored at –80°C until ready for further processing.

DNA was extracted from one aliquot of each strain. After thawing, the cells were suspended in 12 mL 10 mM Tris-EDTA, pH 9.0, containing 50 mg/mL lysozyme, and the cell suspension was transferred to 40 tubes for extraction on the Qiagen BioRobot M48 Workstation (Qiagen Inc., Valencia, CA; Cat. No. 9000708). One "blank" tube containing water was included in each row of the M48 layout to detect any carryover between runs. The blanks must exhibit no DNA signature to meet the acceptance criteria for that run for use in the DNA repository. All 48 tubes (samples and blanks) were processed in the M48 extraction procedure, which includes enzymatic and chemical disruption of cells followed by magnetic bead-based DNA purification.

Upon completion of the extraction procedure, the DNA from the 40 tubes of one strain was pooled and filter-sterilized using a 0.8 μm filter (Corning Inc., Corning, NY) to remove spores with limited shearing of high MW DNA. The filtered DNA was tested for sterility by plating 10% of the extract volume onto 5% sheep blood agar to monitor for growth of viable organisms/spores in the filtered DNA. Filtered DNA showing no growth in the sterility test was subjected to further characterization. Filtered DNA failing the sterility test was refiltered and retested.

DNA characterization included quantity and quality assessments by spectrophotometry (260 nm/280 nm ratios), PicoGreen® (Invitrogen/Molecular Probes Inc., Grand Island, NY) analysis, agarose gel analysis, and characterization by molecular assays.

Spectrophotometry was used to assess the contamination of the DNA with protein. Spectrophotometer readings must indicate an $A_{260/280}$ ratio of ≥1.8. Spectrophotometer readings were not used to quantify DNA because they include all nucleic acids present in the preparation.

PicoGreen analysis detects double-stranded DNA and was used to quantify the DNA. Lambda phage, a double-stranded DNA virus, was used as a control in the spectrophotometer and in the PicoGreen analysis methods. Lambda phage DNA is quantified by the manufacturer, and the PicoGreen reading must approximate the quantification value from the manufacturer for the quantification reading of the DNA to be acceptable.

Agarose gels were used to visualize the DNA preparations. Good preparations showed a large MW band with some smear in the high MW range. Occasionally, the smear extended to the low MW range; this was acceptable if the high MW band was still dominant.

Characterization by molecular assays developed at MRI-FL for *B. anthracis* signatures was used to confirm the presence or absence of signature in the DNA preparations. These assays were developed internally and validated against the *B. anthracis* and near neighbor collection at MRI-FL. These assays were used for QC of spore preparations and DNA, as well as QC assays for assessing panels prior to testing in the MD laboratory.

Each inclusivity organism DNA extract was diluted to 200 000 GE/mL (1.1 ng/mL) in molecular biology grade (MBG) water, and each exclusivity organism DNA extract was diluted to 11.0 ng/mL in MBG water. A set containing one 10 μL aliquot of each DNA preparation was randomized and blind-coded. The sample set was shipped to ITI where each sample was diluted 100-fold in control buffer to ensure the appropriate RAZOR buffer concentration before testing. Each DNA sample was tested once by the RAZOR Anthrax System unless retesting was required by the combination of target calls (*see Results Interpretation*).

Results.—The inclusivity and exclusivity data are summarized in Tables 4 and 5, respectively. The RAZOR Anthrax System inclusivity testing results matched the expected results for all 15 strains of *B. anthracis*, including one strain missing pXO1 and two missing pXO2. For exclusivity, 17 of the 20 strains showed no detection of pXO1, pXO2, or genome, as expected. *B. cereus* G9241 contains a plasmid designated pBCXO1, which is similar, but not identical to, pXO1. Testing of this

Table 4. Inclusivity results[a]

					Plasmid status		RAZOR EX results			
	Strain	Cluster	Strain reference No.	Origin	pXO1	pXO2	pXO1	pXO2	Chromosome	Interpreted result
BA1	Canadian bison	A1a	107448	Bison (Canada)	+	+	+	+	+	vBA[b] detected
BA2	V770-NP-1R	A3a	107240	Vaccine (U.S.)	+	–	+	–	+	pXO1 and BA[c] genome detected
BA3	PAK-1	A2	107518	Sheep (Pakistan)	+	+	+	+	+	vBA detected
BA4	BA1015	A3a	107446	Cow (U.S.–MD)	+	+	+	+	+	vBA detected
BA5	Ames	A3b	107517	Cow (U.S.–TX)	+	+	+	+	+	vBA detected
BA6	K3	A3c	107497	South Africa	+	+	+	+	+	vBA detected
BA7	Ohio ACB	A3d	107339	Pig (U.S.–OH)	+	+	+	+	+	vBA detected
BA8	SK-102	A4	107449	Imported wool (Pakistan)	+	+	+	+	+	vBA detected
BA9	Vollum 1B	A4	107539		+	+	+	+	+	vBA detected
BA10	BA1035	B1	107451	Human (South Africa)	+	+	+	+	+	vBA detected
BA11	RA3	B2	107520	Bovine (France)	+	+	+	+	+	vBA detected
BA12	2002013094 (240)	C	124030	U.S.–LA	+	+	+	+	+	vBA detected
BA13	Pasteur	A1a	107171		–	+	–	+	+	pXO2 and BA genome detected
BA14	Sterne	A3b	107453		+	–	+	–	+	pXO1 and BA genome detected
BA15	Turkey No. 32	A1b	107255	Human (Turkey)	+	+	+	+	+	vBA detected

[a] All inclusivity strains were tested at 2000 GE/mL.

[b] vBA = Virulent *B. anthracis.*

[c] BA = *B. anthracis.*

strain on the RAZOR Anthrax System resulted in detection of pXO1, indicating that the target pXO1 sequence is contained in the pBCXO1 plasmid. *B. cereus* strains 03BB102 and 03BB108 contain incomplete pXO1 plasmids. The pXO1 results on the RAZOR EX were negative for pXO1 in both strains, indicating that the target pXO1 sequence is not found in either incomplete plasmid. These same *B. cereus* strains also contain a plasmid harboring the capA, capB, and capC genes, which are found on pXO2 in *B. anthracis*. The pXO2 results were positive for both *B. cereus* strains, indicating that the target pXO2 sequences are also found on the *B. cereus* plasmids, as expected. *B. cereus* strains 03BB102 and 03BB108 are easily distinguished from *B. anthracis* because the chromosomal marker is negative. Thus, there were no unexpected results in the inclusivity/exclusivity study.

Matrix Studies

The RAZOR Anthrax System was tested with liquid (PBS) and filter matrixes. Each matrix was tested as a clean matrix and loaded with a predetermined amount of standardized dust. Thus, there were four matrixes. Ninety-six replicates of each matrix were inoculated with *B. anthracis* Ames spores at the AMDL, and 96 replicates were inoculated with *B. cereus* E33L spores at 10x AMDL as a negative control. *B. cereus* E33L was chosen as the negative control because it is the closest relative of *B. anthracis*. The acceptance criteria determined by SPADA are: (*1*) no more than one unexpected result allowed out of 96 replicates of *B. anthracis* Ames tested to yield an estimated 5% lower confidence limit on the POD of 0.95 or higher at the AMDL with clean filters or collection liquid; and (*2*) no more than one unexpected result allowed out of 96 replicates of *B. cereus* E33L tested to yield an estimated upper 95% confidence limit on the POD of 0.05 or lower. The acceptance criteria are based on one-sided confidence limits.

Methodology.—Spores were prepared by inoculating a 100 mL starter culture of TSB or BHI with a single frozen aliquot of the appropriate organism. The starter culture was shaken at 120 rpm and 37°C overnight. The overnight starter culture was split into two 50 mL aliquots and added to two separate 1.5 L volumes (3 L total) of a medium appropriate for sporulation. G medium is typically used, but Schaeffer's or Casein acid digest (CAD) may be used for strains not responding well to G medium. The sporulation cultures were shaken at 185 rpm and 37°C until a sporulation level of approximately 90–100% was achieved, as determined by phase contrast microscopy. Spores were then purified by washing three times with cold, sterile water, and resuspended to a final volume of 800 mL in cold, sterile water.

To quantify the spores, serial 10-fold dilutions were made of the spore suspension, and 100 µL of each suspension of the $1 \times 10^{-5, -6, -7}$ dilutions were plated in triplicate on tryptic soy agar (TSA) + 5% sheep blood. Plates were incubated overnight at 37°C. Colonies were counted on plates having between 30

Table 5. Exclusivity results[a]

			Plasmid status		RAZOR EX results			
	Species	Strain	pXO1	pXO2	pXO1	pXO2	Chromosome	Interpreted result
BANN1	*B. cereus*	S2-8	–	–	–	–	–	BA[b] not detected
BANN2	*B. cereus*	3A	–	–	–	–	–	BA not detected
BANN3	*B. thuringiensis*	HD1011	–	–	–	–	–	BA not detected
BANN4	*B. thuringiensis*	97-27	–	–	–	–	–	BA not detected
BANN5	*B. thuringiensis*	HD682	–	–	–	–	–	BA not detected
BANN6	*B. cereus*	E33L	–	–	–	–	–	BA not detected
BANN7	*B. cereus*	D17	–	–	–	–	–	BA not detected
BANN8	*B. thuringiensis*	HD571	–	–	–	–	–	BA not detected
BANN9	*B. cereus*	Al Hakam	–	–	–	–	–	BA not detected
BANN10	*B. cereus*	ATCC 4342	–	–	–	–	–	BA not detected
BANN11	*B. cereus*	FM1	–	–	–	–	–	BA not detected
BANN12	*B. cereus*	G9241	+[c]	–	+	–	–	pXO1 detected
BANN13	*B. cereus*	03BB102	+[d]	+[e]	–	+	–	pXO2 detected
BANN14	*B. cereus*	03BB108	+[d]	+[e]	–	+	–	pXO2 detected
BANN15	*B. thuringiensis*	subspecies *Israelensis* HD 1002	–	–	–	–	–	BA not detected
BANN16	*B. thuringiensis*	subsp. *Kurstaki* HD 1	–	–	–	–	–	BA not detected
BANN17	*B. thuringiensis*	subsp. *Morrisoni* HD 600	–	–	–	–	–	BA not detected
BANN18	*B. coagulans*	ATCC 7050	–	–	–	–	–	BA not detected
BANN19	*B. mycoides*	ATCC 6462	–	–	–	–	–	BA not detected
BANN20	*B. megaterium*	ATCC 12872	–	–	–	–	–	BA not detected

[a] All exclusivity organisms were tested at 20 000 GE/mL.

[b] BA = *B. anthracis.*

[c] pBCXO1 is pXO1-like, but not identical.

[d] Not all of the pXO1 plasmid is present in these two isolates. *B. cereus* 03BB102 is missing parts of the plasmid near ORF 12 through 37, near ORF 84 through 89, and near ORF 115 among others, but is *lef*+, *pagA*+, and *cya*+. *B. cereus* 03BB108 is missing even more extensive regions of pXO1, including the *lef, pagA,* and *cya* genes. The RAZOR Anthrax System pXO1 assay target site is missing in these plasmids.

[e] Cap A, B, and C are contained within the pXO2 plasmid of *B. anthracis* and are found in *B. cereus* strains 03BB102 and 03BB108.

and 300 colonies, the numbers of colony forming units (CFUs) in the countable range were averaged, and the average count and SD were recorded and placed on labels of stock tubes. Eighty milliliters of sterile glycerol was added to the approximately 800 mL spore suspension to achieve a final glycerol concentration of approximately 10%. This suspension was mixed thoroughly and dispensed as follows: (*1*) twenty 30 mL volumes into 50 mL tubes for storage of seeding material; (*2*) one hundred 1 mL volumes into cryovials for storage of primary stocks; (*3*) one hundred 1 mL volumes into cryovials for storage of secondary stocks; and (*4*) twenty-five 1 mL volumes into cryovials for storage as "QA stocks." All stocks were stored at –80°C until needed for downstream processes (i.e., monthly viability testing, spiking, expansion, etc.).

Inoculated clean Lockheed Martin DFU-P-24 (47 mm) filters were prepared by diluting *B. anthracis* Ames spores to 200 000 spores/mL in 50% ethanol (EtOH) and inoculating each of 96 filters with 100 µL of diluted spores, yielding 20 000 spores/filter. Filters were allowed to dry. Another set of 96 clean filters was inoculated with 100 µL *B. cereus* E33L spores diluted to 2×10^6 spores/mL in 50% EtOH. The 96 replicates of *B. anthracis* Ames clean filters and 96 replicates of *B. cereus* E33L clean filters were randomized together, and all samples were blind-coded.

Dusty filters were prepared by loading each filter with 0.1 g standardized dust using a dust delivery device developed at MRI. An aliquot of dust (50 g) was dispensed into a mixing chamber and attached to the dust delivery device. Nitrogen bursts aerosolized the dust into an air column above the dry filter. The geometry of the column and velocity of the dust suspended in the delivery air insured uniform dispersal of the dust in the column. Dust was collected onto the filters at the bottom of the column. The quantity of standardized dust loaded on the filters was based on the average mass of material that was observed to

Table 6. Matrix study results

Matrix	Organism	Concentration	*n*	Target signature detected			X[a]	POD[b]	95% CI[c]	
				pXO1	pXO2	Chromosome			LCL[d]	UCL[e]
Clean filter	*B. anthracis*	20000 spores/filter	96	96	96	94	94	0.98	0.927	0.994
	B. cereus	200000 spores/filter	96	0	0	0	0	0	0.00	0.038
Dust-loaded filter	*B. anthracis*	20000 spores/filter	96	96	96	96	96	1	0.962	1
	B. cereus	200000 spores/filter	96	0	1	0	0	0	0.00	0.038
Clean PBS	*B. anthracis*	2000 spores/mL	96	96	96	96	96	1	0.962	1
	B. cereus	20000 spores/mL	96	0	0	0	0	0	0.00	0.038
Dust-loaded PBS	*B. anthracis*	2000 spores/mL	96	96	96	96	96	1	0.962	1
	B. cereus	20000 spores/mL	96	0	0	0	0	0	0.00	0.038

[a] X = Number of replicates yielding all three targets positive.

[b] POD = Probability of detection.

[c] CI = Confidence interval.

[d] LCL = Lower confidence limit.

[e] UCL = Upper confidence limit.

be collected in repeated 24 h outdoor dry filter unit air filtrations. Ninety-six replicate dusty filters were each inoculated with 100 µL of *B. anthracis* Ames spores at 200 000 spores/mL in 50% EtOH. Another set of 96 dusty filters was inoculated with 100 µL *B. cereus* E33L spores diluted to 2×10^6 spores/mL in 50% EtOH. The 96 replicates of *B. anthracis* Ames dusty filters and 96 replicates of *B. cereus* E33L dusty filters were randomized together and all samples were blind-coded.

To simulate liquid air collection samples at the AMDL, *B. anthracis* Ames spores were diluted to 2000 spores/mL in PBS, and *B. cereus* E33L spores were diluted to 20 000 spores/mL in PBS. Ninety-six replicates of each organism were randomized together, and all samples were blind-coded.

A second set of simulated liquid air collection samples was prepared using 2000 *B. anthracis* Ames spores/mL and 20 000 *B. cereus* E33L spores/mL in PBS charged with 1 mg/mL standardized dust. The level of standardized dust added to the liquid samples was based on the typical shorter collection times with liquid air collectors relative to dry filter units. Ninety-six replicates of each organism with standardized dust were randomized together, and all samples were blind-coded.

Matrix Study testing was performed at MRI-KC under the direction of ITI.

Results.—Each matrix set was analyzed by the RAZOR Anthrax System, and the presence or absence of each target signature determined. Based on the interpretation of the target signature results, the number of correct outcomes was determined for each organism and each matrix. The POD values and associated two-sided 95% confidence intervals were calculated from the number of correct outcomes.

Table 6 summarizes the results of the matrix studies. The results of each target signature are shown as well as the number of correct outcomes (X). The pXO1 target signature yielded the expected results for all replicates of each organism in all matrixes. The pXO2 signature yielded expected results in all cases, except for one replicate of a plasmid-negative *B. cereus* (strain E33L) on dust-loaded filters. The chromosomal signature yielded expected results for all replicates in three of the matrixes. For clean filters, there were two unexpected results in the *B. anthracis* replicates. This yielded a POD value of 0.98 and did not meet the acceptance criteria of no more than one unexpected result in 96 replicates.

Environmental Interference

The environmental interference study was designed to evaluate the effect of environmental substances on the performance of the RAZOR EX method. Potential interfering substances include five soil types, and 23 powders and chemicals, as determined by SPADA. Soils are sources of diverse microbial populations that could potentially cross-react and can contain PCR inhibitors. The 23 powders and chemicals represent common "white powder" substances that could contain PCR inhibitors. Interference can be positive (false positive results) or negative (inhibition of target detection).

All testing was done with solutions or suspensions of potential interfering substances, with either *B. anthracis* added at the AMDL or *B. cereus* added at 10x AMDL. Two types of solutions or suspensions were prepared for each interfering substance: one simulating samples after recovery from filter matrixes into a liquid collection buffer; and another representing liquid samples obtained directly from a liquid collection system. Due to the typical difference in sample collection times for a dry filter unit versus a liquid air collection system, filters are expected to have higher levels of interfering substances. Therefore, a method claiming filters as a matrix is challenged at a higher level of interfering substances relative to a liquid matrix claim. The results are for informational purposes only and do not have any associated acceptance criteria.

Methodology.—Five soil types (sandy, loam, clay, subsoil, and silt) were collected from five diverse locations in the United States (El Paso, TX; Houston, TX; Washington, DC; farmland around Kansas City, KS; and Seattle, WA). Subsoil is defined as material residing between the organic-rich topsoil horizon and the bedrock. Samples were pooled by soil type after prescreening for biothreat signatures using MRI-FL PCR extraction and amplification methods. In addition, after pooling, soils were characterized by standard soil analysis

Table 7. Environmental interference results

Interfering substances	Concentration	Organism	Concentration (spores/mL)	RAZOR EX results			Interpretation
				pXO1	pXO2	Chromosome	
Loam	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected[a]
		B. cereus	20000	–	–	–	BA not detected[b]
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Sandy loam	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Clay	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Subsoil	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Silt	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
B. thuringiensis powder	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered milk	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered infant formula, high Fe	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered infant formula, low Fe	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered coffee creamer	0.1 mg/mL	*B. anthracis*	2,000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected

Table 7. (*continued*)

Interfering substances	Concentration	Organism	Concentration (spores/mL)	RAZOR EX results			Interpretation
				pXO1	pXO2	Chromosome	
Powdered coffee creamer (*contd*)	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered sugar	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Talcum powder	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Wheat flour	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Baking soda	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Chalk dust	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Brewer's yeast	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Dry wall dust	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Cornstarch	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Baking powder	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
γ-Aminobutyric acid	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected

Table 7. (*continued*)

Interfering substances	Concentration	Organism	Concentration (spores/mL)	RAZOR EX results			Interpretation
				pXO1	pXO2	Chromosome	
γ-Aminobutyric acid (*contd*)	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
L-Glutamic acid	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Kaolin	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Chitin	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Chitosan	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
$MgSO_4$	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Boric acid	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20,000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered tooth-paste	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Popcorn salt	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA Detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected

[a] vBA = Virulent *B. anthracis.*

[b] BA = *B. anthracis.*

Table 8. Estimate of LLOD and ULOD

		Target signatures										
		pXO1			pXO2			Chromosome				
			Cp value[a]			Cp value			Cp value			
Purified nucleic acid, mass/test volume	*n*	x	Mean	SD	x	Mean	SD	x[b]	Mean	SD	X[c]	LOD
10 ng	5	5	24.23	0.259	5	22.53	0.193	5	24.13	0.122	5	ULOD
1 ng	5	5	28.27	0.252	5	26.30	0.350	5	27.66	0.301	5	
100 pg	5	5	32.09	0.372	5	30.11	0.182	5	31.57	0.098	5	
10 pg	5	5	35.53	0.376	5	33.61	0.452	5	35.34	0.249	5	
1 pg	5	5	36.77	0.369	5	36.61	0.488	5	38.48	0.462	5	
100 fg	20	20	37.78	1.777	20	39.34	0.701	20	41.96	0.987	20	
50 fg	20	20	38.48	0.451	20	40.18	0.412	20	43.64	0.772	20	LLOD
10 fg	20	18	38.16	0.483	19	39.98	0.621	10	43.65	2.423	9	

[a] Cp = Crossing point.

[b] x = Number of replicates positive for an individual target signature.

[c] X = Number of replicates positive for all three target signatures.

methods by U.S. Department of Agriculture (USDA) Natural Resources Conservation Services. The soil pools were stored and maintained as a standard soil set by MRI-FL.

Soil suspensions were prepared at 0.1 g/mL in PBS for the filter matrix claim and at 7 mg/mL in PBS for the liquid sample claim. Two aliquots of each soil at each concentration were provided to the testing laboratory (MRI-KC).

Twenty-three powders and chemicals from the SPADA environmental factors panel were acquired and stocked at MRI-FL as a standard set. Suspensions were prepared at 0.1 mg/mL in PBS-Triton for the filter matrix claim and at 7 µg/mL in PBS for the liquid sample claim. Two aliquots of each powder and chemical at each concentration were provided to the testing laboratory (MRI-KC).

Blind-coded suspensions of *B. anthracis* Ames spores and *B. cereus* E33L spores were provided separately to the testing laboratory (MRI-KC). Just prior to testing, the soil, powder, and chemical suspensions were spiked with either blinded *B. anthracis* Ames spores at 2000 spores/mL or blinded *B. cereus* E33L spores at 20 000 spores/mL by an operator not involved in sample processing, according to instructions from MRI-FL. The same operator then randomized and blind-coded the spiked samples before providing them to the team of operators processing that day's samples.

Results.—The results are shown in Table 7. There were no false-positive or false-negative results for any of the target signatures in the presence of any of the potential interfering substances.

Upper and Lower LODs of DNA

This study was carried out at ITI and is intended to determine the DNA concentration range that yields reliable results in the PCR assay. The results are for informational purposes only and do not have any associated acceptance criteria.

Methodology.—DNA was isolated from *B. anthracis* Ames using the same procedure as for inclusivity. Ten-fold serial dilutions in control buffer were prepared starting at 10 ng/test volume and ending at 10 fg/test volume. An additional concentration at 50 fg/test volume was chosen by ITI. Dilutions were performed in control buffer so that the final sample contained the appropriate concentration of buffer components for the RAZOR Anthrax System. The test volume, the volume of test material/PCR reaction, is 100 µL for each of the three RAZOR EX PCR assays. Five replicates of each concentration between 10 ng/test volume and 1 pg/test volume were tested in the RAZOR EX PCR assay. Twenty replicates each of 100 fg/test volume, 50 fg/test volume, and 10 fg/test volume were also tested in the RAZOR EX PCR assay.

The lower LOD (LLOD) and upper LOD (ULOD) were

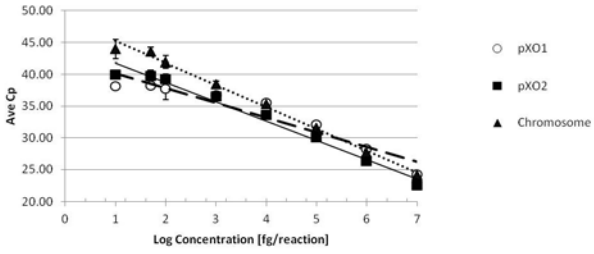

Figure 2. ULOD and LLOD determination linearity of response of the three assays in the RAZOR EX pouch. Dashed line and open circles represent the pXO1 target assay (y = –2.3171x + 42.493, R^2 = 0.9133); solid line and solid squares represent the pXO2 target assay (y = –3.0418x + 44.824, R^2 = 0.9784); and dotted line and solid triangles represent the chromosomal target assay (y = –3.4544x + 48.6543, R^2 = 0.9934).

Table 9. Assay performance at the estimated LLOD and ULOD

				Target signature detected					95% CI[c]	
LOD	Organism	Concentration	*n*	pXO1	pXO2	Chromosome	X[a]	POD[b]	LCL[d]	UCL[e]
ULOD	*B. anthracis*	10 ng/test volume	96	96	96	96	96	1.00	0.96	1.00
LLOD (1)	*B. anthracis*	100 fg/test volume	96	96	96	95	95	0.99	0.94	1.00
LLOD (2)	*B. anthracis*	50 fg/test volume	96	95	95	96	94	0.98	0.93	0.99

[a] X = Number of replicates yielding all three target signatures positive.

[b] POD = Probability of detection.

[c] CI = Confidence interval.

[d] LCL = Lower confidence limit.

[e] UCL = Upper confidence limit.

estimated as the lowest and highest DNA concentrations, respectively, that yielded all replicates positive for all three target signatures. DNA samples were then prepared at these two concentrations, and 96 replicates of each concentration were tested by PCR to confirm the LLOD and ULOD.

Results.—Table 8 shows the results of the initial experiment estimating the LLOD and ULOD. The lowest level yielding all replicates positive for all signatures was 50 fg/test volume. The highest level yielding all replicates positive for all signatures was 10 ng/test volume, which was the highest level tested.

The mean crossing point (Cp) values for each target signature were plotted against the $\log_{10}$ concentration in Figure 2. Over the range tested, the target assays showed a linear response with some variation in slope. The correlation coefficients (R^2 values) were 0.91 for pXO1, 0.98 for pXO2, and 0.99 for the chromosomal assay.

To evaluate the assay performance at the estimated LLOD and ULOD, 96 replicates each of 10 ng/test volume and 50 fg/test volume were tested. An additional level, 100 fg/test volume, was added to ensure that an LLOD meeting the criteria of no more than one unexpected result allowed out of 96 replicates of *B. anthracis* Ames tested to yield an estimated 5% lower confidence limit on the POD of 0.95 or higher would be found. These results are presented in Table 9. The ULOD level was confirmed as having acceptable performance. The true ULOD may be higher than 10 ng/test volume, but this was the highest level tested. For the LLOD, the estimated value of 50 fg/test volume yielded two replicates in which only two out of the three target signatures were detected. One replicate had no detection on the pXO1 assay and the other replicate had no detection on the pXO2 assay, so although the individual assays performed acceptably at 50 fg/test volume, the combined results did not. At twice the DNA concentration, 100 fg/test volume, the combined results were acceptable with only one miss on the chromosomal assay. Therefore, the LLOD was confirmed reliable at 100 fg/test volume.

Robustness

Robustness is the ability of the method performance to remain unaffected by small, deliberate variations in method parameters that can be influenced by the end user. Four parameters were tested in a randomized screening experimental design: storage time of the lysed sample in the bead tube (0 and 2 h at 4 ± 2°C), storage time of the purified sample prior to pouch loading (0 and 4 h at 4 ± 2°C), storage time of the pouch prior to loading the sample [0 and 30 min at room temperature (RT), and storage time of loaded pouches prior to PCR analysis (0 and 30 min at RT)].

Methodology.—*B. anthracis* Ames spores were prepared at the AMDL and at 10x AMDL in PBS and *B. cereus* E33L spores were prepared at 10x AMDL in PBS. Each factorial combination was tested by either two or three replicates of each spore sample (Table 10).

Results.—Table 10 shows the Cp values and maximum fluorescence (MF) values for each target signature and each replicate examined. There were no false positive detections in any of the *B. cereus* reactions.

An analysis of variance (ANOVA) test was performed on the data set to determine whether the variability in the data could be attributed to any single robustness parameter or parameter combination. Parameter 4 (storage time of hydrated pouch prior to PCR analysis) was found to be the only significant contributor to the variability observed in the data set (Table 11). Parameter 4 had a significant adverse effect on the Cp and MF of the pXO1-targeting assay (Table 11). It also had an adverse effect on the MF of the pXO2-chromosome-targeting assays. No other single parameter or parameter combination were found to be significant.

Although Parameter 4 was found to have an adverse effect on the performance of the RAZOR EX Anthrax Air Detection System, it is noteworthy to observe that the presence of *B. anthracis* in the test sample was always correctly identified by the system regardless of the robustness parameter or combination being tested.

Product Consistency and Stability

This study was designed to evaluate the reliability of the products between manufacturing lots and over the course of the product shelf-life. The consumable components of the RAZOR Anthrax System are a DNA extraction kit based on ITI's IT 1-2-3™ Platinum Path Sample purification kit (referred to hereafter as the DNA extraction kit) and a freeze-dried PCR reagent kit contained in a plastic cartridge (the RAZOR pouch). All components of the DNA extraction kit, namely the medium Bead tube, MagBead strip tube, and the antifoam, as well as the

Table 10. Summary of robustness study data

	Parameters						pXO1		pXO2		Chromosome	
Factorial combination	Lysed sample storage, h	Purified sample storage, h	Pouch storage, unloaded, min	Pouch storage, loaded, min	Organism/AMDL level	*n*	Cp[a]	MF[b]	Cp	MF	Cp	MF
1	0	0	0	0	BA@10x AMDL[c]	2	32.79	381.40	32.23	266.20	30.65	169.00
							33.19	395.40	33.79	366.8	35.57	964.8
2	0	0	0	0	BA@AMDL	2	37.18	416.60	36.04	293.20	37.13	612.20
							39.58	433.20	38.46	302.2	40.21	811.6
3	0	0	0	0	BC@10x AMDL[d]	2	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
4	0	0	30	30	BA@10x AMDL	2	35.71	498.2	34.16	335.4	35.89	797.2
							33.81	345.20	33.11	226.8	34.87	657.6
5	0	0	30	30	BA@AMDL	2	36.46	161.4	37.51	249.8	37.74	372.8
							34.59	112.40	36.71	326.2	39.17	786.6
6	0	0	30	30	BC@10x AMDL	2	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
7	0	4	0	30	BA@10x AMDL	2	35.12	480	34.42	340.6	35.12	782.8
							31.08	114.20	32.29	193.40	33.68	510.40
8	0	4	0	30	BA@AMDL	2	37.22	226.00	38.03	287.8	37.3	292.6
							36.55	87.60	36.94	263.00	39.56	527.00
9	0	4	0	30	BC@10x AMDL	2	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
10	0	4	30	0	BA@10x AMDL	2	34.79	460.80	32.46	232	34.07	689.4
							36.47	505.20	35.32	352.4	36.69	874
11	0	4	30	0	BA@AMDL	2	37.31	421.40	36.21	282.20	37.92	708.40
							38.55	314.60	38.13	363.4	40.56	854.4
12	0	4	30	0	BC@10x AMDL	2	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
13	2	0	0	30	BA@10x AMDL	2	34.00	466.60	33.01	284.2	34.54	827.8
							35.07	594.8	33.8	361.8	35.25	901.6
14	2	0	0	30	BA@AMDL	2	35.70	263.00	35.22	217.20	36.73	410.00
							36.68	306.60	37.3	315	39.24	972.2
15	2	0	0	30	BC@10x AMDL	2	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
16	2	0	30	0	BA@10x AMDL	2	36.52	617.60	33.76	327	32.9	303.8
							34.22	365.00	32.83	256	34.91	764.6
17	2	0	30	0	BA@AMDL	2	39.02	397.2	38.79	284	40.1	631.8
							36.71	346.60	36.57	318.00	38.57	802.40
18	2	0	30	0	BC@10x AMDL	2	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
19	2	4	0	0	BA@10x AMDL	3	34.74	414.60	32.70	216.20	34.34	652.80
							37.31	310.2	36.26	351	37.34	823
							35.74	405.8	35	303	36.86	944

Table 10. (*continued*)

	Parameters						pXO1		pXO2		Chromosome	
Factorial combination	Lysed sample storage, h	Purified sample storage, h	Pouch storage, unloaded, min	Pouch storage, loaded, min	Organism/AMDL level	*n*	Cp[a]	MF[b]	Cp	MF	Cp	MF
20	2	4	0	0	BA@AMDL	3	38.69	556.40	37.03	337.8	38.66	683.8
							35.93	136.6	36.82	284	40.28	888
							39.03	122.00	39.11	269	40.74	710
21	2	4	0	0	BC@10x AMDL	3	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
22	2	4	30	30	BA@10x AMDL	3	33.53	554.60	32.26	352.40	33.81	1046.4
							35.31	414.80	34.43	253.80	36.28	797.00
							34.96	579.80	33.90	372.6	35.33	982.4
23	2	4	30	30	BA@AMDL	3	36.08	247.20	36.65	219.00	37.22	436.20
							36.69	280.60	36.89	291.20	39.21	639.20
							36.77	134.8	37.25	224.4	40.09	700.8
24	2	4	30	30	BC@10x AMDL	3	Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
							Neg	Neg	Neg	Neg	Neg	Neg
25	2	4	30	0	BA@10x AMDL	3	35.80	494.40	33.12	253.20	35.23	703.80
							36.44	627.00	34.91	379.8	36.97	1036
							35.06	432.40	33.43	256.4	35.32	744.2
26	2	4	30	0	BA@AMDL	3	38.81	438.60	37.56	269.40	39.09	888.00
							37.61	288.8	38.44	297.2	40.99	850.8
							37.68	293.60	36.49	260.6	39.9	773.6
27	2	4	0	30	BA@10x AMDL	3	34.69	497.40	34.09	332.6	35.95	1107.8
							34.72	453.00	35.30	377.6	36.57	893.4
							35.18	338.20	33.19	181.00	36.17	549.20
28	2	4	0	30	BA@AMDL	3	36.75	245.6	37.54	262.6	37.94	402
							37.23	269.80	38.21	270.40	40.24	718.00
							35.81	176.60	36.77	246	38.67	706.4

[a] Cp = Crossing points.

[b] MF = Maximum fluorescence.

[c] BA = *B. anthracis* Ames.

[d] BC = *B. cereus* E33L.

Table 11. Significant findings from ANOVA of robustness data

Parameter No.	Target/response variable	Organism level	*P*-value of ANOVA
4 Hydrated storage	pXO1/Cp	AMDL	0.0003
4 Hydrated storage	pXO2/MF	AMDL	0.0045
4 Hydrated storage	pXO1/MF	AMDL	0.0119
4 Hydrated storage	Chromosome/MF	AMDL	0.0165

Table 12. DNA extraction kit: Lot-to-lot consistency and stability testing results

			pXO1					pXO2					Chromosome				
				Cp		MF			Cp		MF			Cp		MF	
Platinum path lot No.	Age of kit, months	Organism/AMDL level	Result	Mean	SD	Mean	SD	Result	Mean	SD	Mean	SD	Result	Mean	SD	Mean	SD
410010	1	*B. cereus* 10X	Neg[a]					Neg					Neg				
		B. anthracis 1X	Pos[b]	37.83	0.88	427.52	82.43.	Pos	36.71	0.90	270.20	33.30	Pos	38.49	0.71	808.04	55.57
		B. anthracis 10X	Pos	35.11	1.66	517.17	147.46	Pos	33.49	0.82	288.17	39.46	Pos	35.14	0.68	829.10	61.76
406510	2	*B. cereus* 10X	Neg					Neg					Neg				
		B. anthracis 1X	Pos	38.27	0.96	436.56	90.87	Pos	36.80	0.76	226.64	15.80	Pos	38.51	0.97	637.84	82.35
		B. anthracis 10X	Pos	35.36	0.69	555.50	26.51	Pos	33.26	0.85	237.20	32.74	Pos	35.01	0.86	712.17	89.55
740210	3	*B. cereus* 10X	Neg					Neg					Neg				
		B. anthracis 1X	Pos	37.49	2.90	423.56	216.34	Pos	36.81	0.60	292.72	38.77	Pos	39.30	0.41	800.88	66.28
		B. anthracis 10X	Pos	35.74	0.89	572.92	18.06	Pos	32.89	2.14	292.76	105.14	Pos	35.57	0.67	888.40	101.82
722610	6	*B. cereus* 10X	Neg					Neg					Neg				
		B. anthracis 1X	Pos	38.56	0.73	542.48	108.77	Pos	37.25	0.42	293.36	37.84	Pos	39.00	0.62	832.20	162.73
		B. anthracis 10X	Pos	35.83	0.79	609.60	34.36	Pos	33.77	0.77	312.24	36.21	Pos	35.52	0.87	937.40	140.60
717010	7	*B. cereus* 10X	Neg					Neg					Neg				
		B. anthracis 1X	Pos	39.14	1.46	441.50	79.22	Pos	37.00	2.67	244.40	92.43	Pos	39.37	1.22	760.07	62.09
		B. anthracis 10X	Pos	36.73	1.79	532.76	25.85	Pos	34.49	1.34	340.80	46.29	Pos	36.12	1.13	888.40	96.89
712709	8	*B. cereus* 10X	Neg					Neg					Neg				
		B. anthracis 1X	Pos	39.04	1.22	393.57	56.16	Pos	37.92	0.93	224.23	35.05	Pos	39.55	1.23	640.73	110.36
		B. anthracis 10X	Pos	36.49	0.56	498.96	64.34	Pos	34.55	0.61	277.40	34.06	Pos	36.29	0.65	786.84	56.57

[a] Neg = Negative.

[b] Pos = Positive.

Table 13. Significant findings from ANOVA to examine lot-to-lot consistency of DNA extraction kit

DNA extraction kit consistency ANOVA results (response variable Cp)		
Target	Organism level	*P*-value of ANOVA
pXO1	1x AMDL	0.436
	10x AMDL	0.209
pXO2	1x AMDL	0.674
	10x AMDL	0.185
Chromosome	1x AMDL	0.322
	10x AMDL	0.098

RAZOR pouches were evaluated. All components were stored as recommended at room temperature.

Methodology.—For evaluation of the DNA extraction kit reagents, *B. anthracis* Ames spores were prepared at 2000 and 20 000 spores/mL in PBS. *B. cereus* E33L spores were prepared at 20 000 spores/mL in PBS. Five replicates of each sample were tested on each of six lots of the DNA extraction kit ranging in age from 1 to 8 months. The testing was performed at MRI-KC.

For evaluation of the RAZOR pouches, *B. anthracis* Ames DNA was prepared at 2000 genome equivalents/mL (11 pg/mL) and 20 000 genome equivalents/mL (110 pg/mL) in control buffer from a 1 ng/µL stock provided by MRI-FL. *B. cereus* E33L DNA was prepared at 20 000 genome equivalents/mL (110 pg/mL) in control buffer from a 0.67 ng/µL stock provided by MRI-FL. Dilutions were made in the control buffer to ensure that the concentration of buffer was appropriate for testing in the RAZOR Anthrax System. Five replicates of each sample were tested on each of six lots of RAZOR EX Anthrax pouches ranging in age from 1 to 6 months. The testing was performed at ITI.

Results.—*DNA extraction kit*.—Table 12 presents the data for the DNA extraction kit. All *B. cereus* samples were negative for all assays. No significant lot-to-lot differences were observed in Cp values for the six lots of DNA extraction kit tested in this study (Table 13). A linear regression analysis was performed to evaluate whether the age of a kit had a significant effect on performance (Table 14). Age of the DNA extraction kit had a significant effect on the Cp value of all assays for *B. anthracis* samples at 10x AMDL. For *B. anthracis* samples at the AMDL, there was a significant effect of age on the Cp value of the pXO1 target assay but not on the other two assays. However, in all cases, the R^2 value is low (<25%) and slopes of the regression equations are very small, yielding a difference of Cp (ΔCp) of less than 1.5 cycles between the oldest and newest lot. This small difference in Cp is not of practical significance. Statistical analyses were performed only on Cp values because they can indicate the level of DNA extracted.

PCR reagent kit (RAZOR pouch).—Table 15 shows the data for the between-lot and stability testing of the PCR pouch reagents. All *B. cereus* samples for all assays were negative. Significant lot-to-lot differences were observed in Cp and MF values for the six lots of RAZOR pouches tested in this study (Table 16). However, the Cp variability between lots was less than ±2 cycles, and the minimum MF was above 200 units. This degree of variability will not affect positive detection. A linear regression analysis was performed to evaluate whether the age of RAZOR pouch had a significant effect on performance (Table 17). Age of the RAZOR pouch did not affect the Cp values of any assays for *B. anthracis* samples at 10x AMDL. For *B. anthracis* samples at the AMDL, there was a significant effect of age on the Cp value of the pXO1 and pXO2 target assays, but not on the chromosomal target assay. However, in all cases, the R^2 is low (<22%), and the slopes of the regression equations are very small, yielding a ΔCp of 1.25 cycles or less between the oldest and newest lot. These differences are of very little practical significance. Age of the RAZOR pouch has a significant effect on the MF values for the pXO2 target assay for *B. anthracis* samples at 10x AMDL or for the chromosomal target assay for *B. anthracis* samples at AMDL and 10x AMDL. However, even in the oldest pouches the MF values of all assays are well above the level required for positive detection.

Instrument Variability

Six RAZOR EX instruments were tested to determine the instrument variability.

Methodology.—*B. anthracis* Ames spores were prepared at 2000 and 20 000 spores/mL in PBS. *B. cereus* E33L spores were prepared at 20 000 spores/mL in PBS. Five replicates of each spore sample were tested on each of six RAZOR EX instruments. The testing was performed at MRI-KC.

Results.—The instrument variability results are presented in Table 18. All *B. cereus* samples gave negative results on all assays. An ANOVA was performed to examine if the instruments had variable performance (Table 19). There was a significant

Table 14. Results of linear regression analyses to examine the stability of DNA extraction kit over time

Organism/AMDL level	Target	Regression equation response variable = Cp	R^2, %[a]	*P* value of regression
B. anthracis 10X	pXO1	35.0 + (0.203 × age of kit)	19.8	0.011
	pXO2	32.9 + (0.189 × age of kit)	16.9	0.02
	Chromosome	34.9 + (0.163 × age of kit)	24.2	0.004
B. anthracis 1X	pXO1	37.5 + (0.195 × age of kit)	11.8	0.054
	pXO2	36.5 + (0.131 × age of kit)	7.2	0.138
	Chromosome	38.4 +(0.132 × age of kit)	13.7	0.037

[a] R^2 = Coefficient of determination (percentage of Cp variation that is explained by the age of the DNA extraction kit).

Table 15. PCR pouch reagents lot-to-lot consistency and stability testing results

			pXO1						pXO2					Chromosome			
				Cp		MF			Cp		MF			Cp		MF	
RAZOR pouch Lot No.	Age of pouch, months	Organism/AMDL level	Result	Mean	SD	Mean	SD	Result	Mean	SD	Mean	SD	Result	Mean	SD	Mean	SD
410010	1	*B. cereus* 10x	Neg[a]					Neg					Neg				
		B. anthracis 1x	Pos[b]	39.99	0.635	373	33.24	Pos	39.62	0.424	276.1	14.23	Pos	40.8	0.317	702	74.96
		B. anthracis 10x	Pos	37.12	0.82	453	92.01	Pos	35.472	0.64	304.88	33.74	Pos	36.48	0.66	789.36	41.13
303310	2	*B. cereus* 10x	Neg					Neg					Neg				
		B. anthracis 1x	Pos	39.91	0.37	414.6	35.94	Pos	39.35	0.491	258.8	37.8	Pos	39.62	0.893	564.8	122.9
		B. anthracis 10x	Pos	37.36	0.165	571.16	26.69	Pos	35.822	0.5	291.56	20.63	Pos	36.69	0.46	745.56	47.29
301210	3	*B. cereus* 10x	Neg					Neg					Neg				
		B. anthracis 1x	Pos	39.14	0.427	524.2	22.34	Pos	38.13	0.554	288.6	34.76	Pos	39.84	0.277	749.8	31.32
		B. anthracis 10x	Pos	35.436	0.5	524.88	81.56	Pos	34.01	0.74	245.52	32.12	Pos	35.594	0.465	727.2	65.24
284010	4	*B. cereus* 10x	Neg					Neg					Neg				
		B. anthracis 1x	Pos	40.33	0.805	370.3	50.84	Pos	39.84	0.679	279	34.54	Pos	41.04	0.567	588	38
		B. anthracis 10x	Pos	37.590	1.309	588.560	23.729	Pos	35.874	1.115	315.120	11.536	Pos	36.974	0.633	689.92	55.349
271409	5	*B. cereus* 10x	Neg					Neg					Neg				
		B. anthracis 1x	Pos	40.89	0.464	324.5	20.09	Pos	40.66	0.44	224.2	25.98	Pos	41.25	0.409	575.5	64.85
		B. anthracis 10x	Pos	37.966	0.275	428.95	29.77	Pos	36.134	0.53	225.1	25.93	Pos	36.738	1	598.7	67.83
265109	6	*B. cereus* 10x	Neg					Neg					Neg				
		B. anthracis 1x	Pos	40.47	0.343	353.7	46.56	Pos	40.25	0.443	249.9	25.58	Pos	40.7	0.411	566.4	56.14
		B. anthracis 10x	Pos	37.31	0.39	476.24	45.83	Pos	35.32	0.43	241.32	42.44	Pos	36.17	0.21	614.44	100.4

[a] Neg = Negative.

[b] Pos = Positive.

effect of the instrument on the Cp value for the pXO1 target assay for *B. anthracis* at the AMDL, but not at 10x AMDL. The instruments were found to not have a significant effect on

Table 18. Between-instrument variability results

		pXO1					pXO2					Chromosome				
			Cp		MF			Cp		MF			Cp		MF	
RAZOR EX No.	Organism/AMDL level	Result	Mean	SD	Mean	SD	Result	Mean	SD	Mean	SD	Result	Mean	SD	Mean	SD
4026	*B. cereus* 10X	Neg[a]					Neg					Neg				
	B. anthracis 1X	Pos[b]	38.05	1.13	437.77	47.18	Pos	37.5	1.22	346.3	31.57	Pos	38.4	1.23	698.33	73.77
	B. anthracis 10X	Pos	36.13	0.66	568.44	28.64	Pos	33.89	0.73	342.84	15.52	Pos	34.75	1.01	718.84	220.05
4051	*B. cereus* 10X	Neg					Neg					Neg				
	B. anthracis 1X	Pos	38.01	1.23	393.92	41.59	Pos	37.21	1.07	286	14.5	Pos	38.32	0.91	732.68	48.43
	B. anthracis 10X	Pos	35.76	0.66	465.44	18.25	Pos	33.87	0.44	297.72	14.4	Pos	35.57	0.36	838.72	19.97
4055	*B. cereus* 10X	Neg					Neg					Neg				
	B. anthracis 1X	Pos	36.34	1.68	379	167.39	Pos	36.19	0.94	391.32	19.21	Pos	37.9	0.92	935.44	90.37
	B. anthracis 10X	Pos	34.47	2.14	534.36	165.03	Pos	33.14	0.42	348.32	31.04	Pos	34.48	0.59	1009.16	179.26
4058	*B. cereus* 10X	Neg					Neg					Neg				
	B. anthracis 1X	Pos	37.85	0.56	451.88	21.25	Pos	36.58	0.74	267.48	7.74	Pos	37.77	0.84	643.48	206.11
	B. anthracis 10X	Pos	34.89	1.48	527.72	18.37	Pos	33	0.72	282.68	12.49	Pos	34.82	0.76	759.72	38.71
4064	*B. cereus* 10X	Neg					Neg					Neg				
	B. anthracis 1X	Pos	38.63	0.6514	473.88	36.0041	Pos	37.55	0.9957	345.36	19.3594	Pos	39	1.0796	813.56	41.0294
	B. anthracis 10X	Pos	36.43	1.1856	611.16	19.3238	Pos	34.25	1.3697	370.16	6.20226	Pos	35.83	1.161	875.76	102.02
4080	*B. cereus* 10X	Neg					Neg					Neg				
	B. anthracis 1X	Pos	38.59	0.49	447.08	16.54	Pos	37.36	0.66	323.53	26.63	Pos	38.33	0.72	719.93	206.95
	B. anthracis 10X	Pos	36.15	1.2	522.36	27.73	Pos	33.95	1.13	354.2	17.68	Pos	35.7	1.15	894.68	46.53

[a] Neg = Negative.
[b] Pos = Positive.

Table 19. Significant findings from ANOVA to examine between-instrument variability

Between-instrument variability ANOVA results			
		P-value of ANOVA	
Target	Organism level	Cp	MF
pXO1	1x AMDL	0.024	0.335
	10x AMDL	0.172	0.093
pXO2	1x AMDL	0.165	0.000
	10x AMDL	0.217	0.000
Chromosome	1x AMDL	0.435	0.026
	10x AMDL	0.130	0.023

matrix was spiked with *B. anthracis* spores at the AMDL and with *B. cereus* E33L at 10x AMDL.

Methodology.—The methodology was the same as for the MD study. See *Method Developer Validation Studies, Matrix Studies, Methodology*.

Results.—Each matrix set was analyzed by the RAZOR Anthrax System, and the presence or absence of each target signature determined. Based on the interpretation of the target signature results, the number of correct outcomes was determined for each organism and each matrix. The POD values and associated two-sided 95% confidence intervals were calculated from the number of correct outcomes. The matrix study results are presented in Table 22. The results of each target signature are shown as well as the number of correct outcomes (X).

For all four matrixes tested, the pXO2 and the chromosomal target signatures were detected in 96 out of 96 replicates spiked with *B. anthracis*, thus meeting the acceptance criterion of 95/96 detections for these signatures. The acceptance criterion for the pXO1 target signature was met for only three of the four matrixes: the clean and dust-loaded PBS, as well as the dust-loaded dry filter units (DFU). In these three matrixes, the pXO1 target signature was detected in 96/96 replicates. In the clean DFU filter matrix, the pXO1 target signature was detected in only 93/96 replicates, which does not meet the acceptance criterion of 95/96. There were no positives for any of the signatures in replicates spiked with *B. cereus* E33L for all four matrixes.

Both the clean and dust-loaded PBS matrixes, as well as the dust-loaded DFU matrix met the overall acceptance criteria for *B. anthracis* detection by giving a POD of 1.00 and a lower confidence limit (LCL) of 0.962. Due to three failures to detect the pXO1 target signature in the clean DFU filter replicates spiked with *B. anthracis*, the overall POD for the clean DFU filter matrix was 0.97 with an LCL of 0.912. This matrix,

Table 20. Independent laboratory inclusivity results[a]

					Plasmid status		RAZOR EX results			
No.	Strain	Cluster	Strain reference No.	Origin	pXO1	pXO2	pXO1	pXO2	Chromosome	Interpreted result
BA1	Canadian bison	A1a	107448	Bison (Canada)	+	+	+	+	+	vBA[b] detected
BA2	V770-NP-1R	A3a	107240	Vaccine (U.S.)	+	–	+	–	+	pXO1 and BA[c] chromosome detected
BA3	PAK-1	A2	107518	Sheep (Pakistan)	+	+	+	+	+	vBA detected
BA4	BA1015	A3a	107446	Cow (U.S.–MD)	+	+	+	+	+	vBA detected
BA5	Ames	A3b	107517	Cow (U.S.–TX)	+	+	+	+	+	vBA detected
BA6	K3	A3c	107497	South Africa	+	+	+	+	+	vBA detected
BA7	Ohio ACB	A3d	107339	Pig (U.S.–OH)	+	+	+	+	+	vBA detected
BA8	SK-102	A4	107449	Imported wool (Pakistan)	+	+	+	+	+	vBA detected
BA9	Vollum 1B	A4	107539		+	+	+	+	+	vBA detected
BA10	BA1035	B1	107451	Human (South Africa)	+	+	+	+	+	vBA detected
BA11	RA3	B2	107520	Bovine (France)	+	+	+	+	+	vBA detected
BA12	2002013094 (240)	C	124030	U.S.–LA	+	+	+	+	+	vBA detected
BA13	Pasteur	A1a	107171		–	+	–	+	+	pXO2 and BA chromosome detected
BA14	Sterne	A3b	107453		+	–	+	–	+	pXO1 and BA chromosome detected
BA15	Turkey No. 32	A1b	107255	Human (Turkey)	+	+	+	+	+	vBA detected

[a] All inclusivity strains were tested at 2000 GE/mL.

[b] vBA = Virulent *B. anthracis*.

[c] BA = *B. anthracis*.

Table 21. Independent laboratory exclusivity results[a]

			Plasmid status		RAZOR EX results			
No.	Species	Strain	pXO1	pXO2	pXO1	pXO2	Chromosome	Interpreted result
BANN1	*B. cereus*	S2-8	–	–	–	–	–	Negative for BA[b]
BANN2	*B. cereus*	3A	–	–	–	–	–	Negative for BA
BANN3	*B. thuringiensis*	HD1011	–	–	–	–	–	Negative for BA
BANN4	*B. thuringiensis*	97-27	–	–	–	–	–	Negative for BA
BANN5	*B. thuringiensis*	HD682	–	–	–	–	–	Negative for BA
BANN6	*B. cereus*	E33L	–	–	–	–	–	Negative for BA
BANN7	*B. cereus*	D17	–	–	–	–	–	Negative for BA
BANN8	*B. thuringiensis*	HD571	–	–	–	–	–	Negative for BA
BANN9	*B. cereus*	Al Hakam	–	–	–	–	–	Negative for BA
BANN10	*B. cereus*	ATCC 4342	–	–	–	–	–	Negative for BA
BANN11	*B. cereus*	FM1	–	–	–	–	–	Negative for BA
BANN12	*B. cereus*	G9241	+[c]	–	+	–	–	pXO1 Detected
BANN13	*B. cereus*	03BB102	+[d]	+[e]	–	+	–	pXO2 Detected
BANN14	*B. cereus*	03BB108	+[d]	+[e]	–	+	–	pXO2 Detected
BANN15	*B. thuringiensis*	subsp. *Israelensis* HD 1002	–	–	–	–	–	Negative for BA
BANN16	*B. thuringiensis*	subsp. *Kurstaki* HD 1	–	–	–	–	–	Negative for BA
BANN17	*B. thuringiensis*	subsp. *Morrisoni* HD 600	–	–	–	–	–	Negative for BA
BANN18	*B. coagulans*	ATCC 7050	–	–	–	–	–	Negative for BA
BANN19	*B. mycoides*	ATCC 6462z	–	–	–	–	–	Negative for BA
BANN20	*B. megaterium*	ATCC 12872	–	–	–	–	–	Negative for BA

[a] All exclusivity strains were tested at 20000 GE/mL.

[b] BA = *B. anthracis.*

[c] pBCXO1 is pXO1-like, but not identical.

[d] Not all of the pXO1 plasmid is present in these two isolates. *B. cereus* 03BB102 is missing parts of the plasmid near ORF 12 through 37, near ORF 84 through 89, and near ORF 115, among others, but is *lef*+, *pagA*+, and *cya*+. *B. cereus* 03BB108 is missing even more extensive regions of pXO1, including the *lef, pagA,* and *cya* genes.

[e] Cap A, B, and C are contained within the pXO2 plasmid of *B. anthracis* and are found in *B. cereus* strains 03BB102 and 03BB108.

Table 22. Independent laboratory matrix study results

				Target signature detected					95% CI[c]	
Matrix	Organism	Concentration	*n*	pXO1	pXO2	Chromosome	X[a]	POD[b]	LCL[d]	UCL[e]
Clean filter	*B. anthracis*	20000 spores/filter	96	93	96	96	93	0.97	0.912	0.989
	B. cereus	200000 spores/filter	96	0	0	0	0	0	0.00	0.038
Dust-loaded filter	*B. anthracis*	20000 spores/filter	96	96	96	96	96	1	0.962	1
	B. cereus	200000 spores/filter	96	0	0	0	0	0	0.00	0.038
Clean PBS	*B. anthracis*	2000 spores/mL	96	96	96	96	96	1	0.962	1
	B. cereus	20000 spores/mL	96	0	0	0	0	0	0.00	0.038
Dust-loaded PBS	*B. anthracis*	2000 spores/mL	96	96	96	96	96	1	0.962	1
	B. cereus	20000 spores/mL	96	0	0	0	0	0	0.00	0.038

[a] X = Number of replicates yielding all three targets positive.

[b] POD = Probability of detection.

[c] CI = Confidence interval.

[d] LCL = Lower confidence limit.

[e] UCL = Upper confidence limit.

Table 23. Environmental substance results from the independent laboratory

				RAZOR EX results			
Interfering substances	Concn	Organism	Concn, spores/mL	pXO1	pXO2	Chromosome	Interpretation
Loam	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA[a] detected
		B. cereus	20000	–	–	–	BA[b] not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Sandy loam	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Clay	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Subsoil*	0.1 g/mL	*B. anthracis*	2000	–	+	+	pXO2 and BA Genome detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Silt	0.1 g/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
B. thuringiensis powder	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered milk	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered infant formula, high Fe	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered infant formula, low Fe	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered coffee creamer	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected

Table 23. (*continued*)

Interfering substances	Concn	Organism	Concn, spores/mL	RAZOR EX results			
				pXO1	pXO2	Chromosome	Interpretation
Powdered sugar	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Talcum powder	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Wheat flour	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Baking soda	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Chalk dust	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Brewer's yeast	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Dry wall dust	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Cornstarch	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Baking powder	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
γ-Aminobutyric acid	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected

Table 23. (*continued*)

Interfering substances	Concn	Organism	Concn, spores/mL	RAZOR EX results			
				pXO1	pXO2	Chromosome	Interpretation
L-Glutamic acid	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Kaolin	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Chitin	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Chitosan	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
$MgSO_4$	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Boric acid	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Powdered toothpaste	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
Popcorn salt	0.1 mg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected
	7 µg/mL	*B. anthracis*	2000	+	+	+	vBA detected
		B. cereus	20000	–	–	–	BA not detected

* pXO1 target signature was not detected in the *B. anthracis* sample in the presence of subsoil at a 0.1 mg/mL concentration.

[a] vBA = Virulent *B. anthracis.*

[b] BA = *B. anthracis.*

Table 24. Environmental organism results from the independent laboratory

		RAZOR EX results			
Pool	Spike	pXO1	pXO2	Chromosome	Interpretation
1	*B. anthracis*, 4000 GE/mL[a]	+	+	+	Positive for BA[b]
	None	–	–	–	Negative for BA
2	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
3	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
4	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
5	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
6	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
7	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
8	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
9[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
10[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
11[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
12[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
13[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
14[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
15[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
16[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
17[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA
18[c]	*B. anthracis*, 4000 GE/mL	+	+	+	Positive for BA
	None	–	–	–	Negative for BA

[a] GE = Genomic equivalents.

[b] BA = *B. anthracis*.

[c] Individual eukaryotes – *see* Appendix 1. Tested at the highest achievable concentration which was lower than 10X AMDL.

Table 25. Upper and lower limits of DNA detection at the independent laboratory

		Target signature										
		pXO1			pXO2			Chromosome				
			Cp value[a]			Cp value			Cp value			
Purified nucleic acid, mass/test volume	*n*	x	Mean	SD	x[b]	Mean	SD	x	Mean	SD	X[c]	LOD
10 ng	5	5	26.63	0.422	5	23.99	0.383	5	24.97	0.325	5	ULOD
1ng	5	5	31.23	0.637	5	28.03	0.487	5	28.85	0.695	5	
100 pg	5	5	34.48	0.265	5	31.45	0.216	5	32.21	0.076	5	
10 pg	5	5	38.55	0.956	5	35.93	0.646	5	36.68	0.852	5	
1 pg	5	5	38.89	1.119	5	39.08	0.222	5	38.84	0.539	5	LLOD
100 fg	20	20	40.74	0.916	20	43.04	1.177	19	42.38	1.101	19	
50 fg	20	14	40.78	1.861	20	43.92	1.464	18	41.89	2.003	12	
10 fg	20	8	40.38	1.292	6	43.87	2.385	11	42.5	2.932	0	

[a] Cp = Crossing point.

[b] x = Number of replicates positive for an individual target signature.

[c] X = Number of replicates positive for all three target signatures.

therefore, did not meet the overall acceptance criteria. All four matrixes met the overall acceptance criteria for *B. cereus* replicates with a POD of 0.00 (0/96) and an upper confidence limit (UCL) of 0.038.

Environmental Interference

The ILV environmental interference study included the five soil types and 23 powders and chemicals as in the MD study. These potentially interfering substances were tested at a low concentration in PBS and a high concentration in filter recovery buffer (PBS with 0.1% Triton X-100) to reflect the difference in typical collection times between DFUs and liquid air collection systems. DFUs are typically used for longer collection times, which results in capture of higher amounts of airborne environmental substances. In addition, the independent laboratory tested the environmental organisms recommended by SPADA. These were tested as DNA extracts at 10x AMDL (expressed as genome equivalents). The prokaryotes and lower eukaryotes were tested as pools of DNA extracts with each individual organism represented at 10x AMDL. The higher eukaryotes were tested as individual DNA extracts. Where testing at 10x AMDL was not possible due to the genome size, the DNA was tested at the highest possible concentration.

Methodology.—The methodology for testing environmental interference from soils, powders, and chemicals was the same as for the MD study. *See Method Developer Validation Studies, Environmental Interference, Methodology*.

To test interference from environmental organism, DNA was prepared from *B. anthracis* and each of the 78 environmental organisms or representative cell lines (*see* Appendix 1) on the Qiagen BioRobot M48 using a lysozyme treatment as in section *Method Developer Validation Studies, Inclusivity and Exclusivity, Methodology*. DNA from the environmental organisms was pooled or tested individually as indicated in Appendix 1. The environmental organism DNA was diluted in the RAZOR Anthrax Air control buffer such that the final concentration of each panel member was 10 times the AMDL (20 000 genome equivalents/mL) wherever possible. Pooled or individual DNA was tested with and without the addition of *B. anthracis* Ames DNA at 2x AMDL (4000 genome equivalents/mL). All samples were randomized, blind-coded, and tested according to the RAZOR Anthrax Air System instructions.

Results.—The results from environmental interference substance testing are presented in Table 23, and the results from environmental interference organism testing are presented in Table 24. The only unexpected result occurred with the high level of subsoil interfering with detection of pXO1. There were no other unexpected results in the presence of environmental substances or organisms.

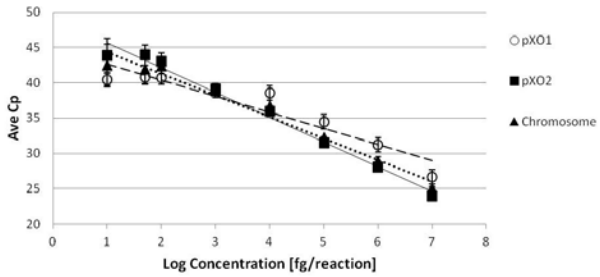

Figure 3. ULOD and LLOD determination linearity of response of the three assays in the RAZOR EX pouch at the independent laboratory. Dashed line and open circles represent the pXO1 target assay ($y = -2.3171x + 42.493$, $R^2 = 0.9133$); solid line and solid squares represent the pXO2 target assay ($y = -3.5283x + 42.263$, $R^2 = 0.9861$); and dotted line and solid triangles represent the chromosomal target assay ($y = -3.0561x + 47.385$, $R^2 = 0.973$).

Table 26. Assay performance in the independent laboratory at the estimated ULOD and LLOD

				Target signature detected					95% CI[c]	
LOD	Organism	Concentration	*n*	pXO1	pXO2	Chromosome	X[a]	POD[b]	LCL[d]	UCL[e]
ULOD	*B. anthracis*	10 ng/test volume	96	96	96	96	96	1.00	0.96	1.00
LLOD	*B. anthracis*	1 pg/test volume	96	96	96	96	96	1.00	0.96	1.00

[a] X = Number of replicates yielding all three target signatures positive.

[b] POD = Probability of detection.

[c] CI = Confidence interval.

[d] LCL = Lower confidence limit.

[e] UCL = Upper confidence limit.

Upper and Lower Limits of DNA Detection

Methodology.—The methodology was the same as for the MD study. *See Method Developer Validation Studies, Upper and Lower LODs of DNA, Methodology*.

Results.—Results of the DNA dilutions series for LOD determination are presented in Table 25. As in the MD study, the highest level tested, 10 ng/test volume, yielded 5/5 replicates positive for all target signatures and serves as the estimated upper limit of detection of DNA. As the DNA level was decreased, the first target to be missed by the system was the chromosomal target at 100 fg/test volume followed by the pXO1 target at 50 fg/test volume. The pXO2 target did not experience a miss until the DNA concentration was down to the lowest level tested, 10 fg/test volume. Therefore, the LLOD is estimated at 1 pg/test volume, where no misses were observed for any of the three targets. This is in contrast to the MD study results, which demonstrated no misses for any of the targets down to and including 50 fg/test volume (*see* Table 8). The mean Cp values for each of the target assays are plotted against the log concentration of the LOD determination series test level in Figure 3. The Cp values for concentrations higher than 100 fg/test volume are linear as plotted, demonstrating consistent PCR efficiency.

Finally, the assay performance was verified at the estimated at the ULOD and LLOD by testing 96 replicates of DNA at each concentration. These results (Table 26) demonstrate acceptable performance at these DNA concentrations with 96/96 replicates detected for each assay at each concentration. Thus, in the independent laboratory, the RAZOR Anthrax assays were reliable at the ULOD of 10 ng/test volume and the LLOD of 1 pg/test volume.

Discussion

The MD and ILV studies demonstrate acceptable performance of the RAZOR EX Anthrax Air Detection System as determined by the SPADA method performance requirements. Inclusivity and exclusivity testing produced expected results for all 15 *B. anthracis* strains and 20 near neighbor exclusivity organisms. The matrix studies showed acceptable performance for clean PBS, dust-loaded PBS, and dust-loaded filter samples. The clean filter matrix did not meet the acceptance criteria; there were two false negatives in the MD study and three false negatives in the ILV study. Only one miss is allowed by the SPADA criteria.

We theorize that the discrepancy in the performance of the two filter matrixes could be attributed to lower spore recovery from clean filters into the extraction buffer due to greater adhesion of spores to clean filters compared to dust-loaded filters. This lower spore recovery may result in a DNA concentration near the LOD of the target PCR reactions. The presence of dust on the filters likely reduces adhesion of the spores to the filter material, resulting in higher spore recovery from dust-loaded filters and, thus, a higher probability of detection of the targets.

In evaluating this method, it is important to consider its intended use, which is to detect *B. anthracis* spores in environmental samples collected from liquid or filter-based air samplers. Concurrent deposition of ambient dust on filters, alongside spores, would be expected from usually dusty sampling environments. Therefore, more emphasis should be placed on results obtained from simulated samples that more accurately represent true environmental samples, in this case the dust-loaded filters. Also, if the outcomes of the clean and dust-loaded filters used in these validations are combined to represent the filter matrix as a whole, then the filter matrix meets the acceptance criteria for both the MD study [POD = 0.99, 95% confidence interval (CI) extends from 0.9604 to 0.9996] and the ILV study (POD = 0.98, 95% CI extends from 0.9530 to 0.9968).

The ability of the RAZOR Anthrax system to provide reliable results in the presence of potentially interfering environmental substances was also tested. Five soil types, and 23 powders and chemicals were tested for both positive and negative interference, and the data demonstrated that none of the substances interfered with any of the assays, with the exception of the high level of subsoil interfering with the pXO1 assay in the ILV study. By definition, subsoil resides between the organic-rich topsoil horizon and the bedrock and, therefore, is not likely to be aerosolized except near a construction or work site where digging is actively taking place. Thus, this interference is of low practical impact. Although these data are for informational purposes only, they demonstrate the ability of the RAZOR Anthrax System to perform well in the presence of a wide variety of potential environmental interfering substances. This is an important characteristic for a method intended to analyze air samples collected indoors or outdoors.

In addition to the soils, powders, and chemicals, the independent laboratory examined interference from DNA of 78 environmental organisms ranging from bacteria found in air and soil to higher eukaryotes (*see* Appendix 1). No interference was observed (no false-positive or -negative results) with any of

the individual or pooled organisms tested, meeting the SPADA acceptance criterion of 100% expected results.

The RAZOR Anthrax System's LOD of DNA was also examined using purified nucleic acid. The system was shown to have a range of reliable detection from 100 fg/reaction volume to at least 10 ng/reaction volume in the MD study, and from 1 pg/reaction volume to at least 10 ng/reaction volume in the ILV study. As the system, including instruments, extraction kits, and pouches, showed consistent performance, the discrepancy between the LLODs of the two studies is probably due to variability in nucleic acid preparations. Based on the ILV results, a conservative estimate of 1 pg/reaction (equivalent to 200 spores/100 mL) for the lower limit of DNA detection can be made. Within the range of 100 pg/reaction volume to 10 ng/reaction volume, the PCR response is linear with the $\log_{10}$ concentration; however, the method is not intended to be quantitative.

Finally, the results of the between-lot consistency and stability studies for both the DNA extraction kit reagents and the RAZOR pouches were found to be well within the range for detection. The stability testing of the RAZOR pouches also demonstrated no loss of vacuum in appropriately stored pouches. The variability between instruments was also shown to be acceptable. Robustness testing demonstrated no effect on results from storage of the lysed samples in bead tubes for up to 2 h at 4°C, storage of the purified sample for up to 4 h at 4°C, or storage of opened pouches before sample loading for up to 30 min at room temperature. There was a significant adverse effect on Cp and MF in pouches stored for 30 min at RT after sample loading before PCR; however, the accuracy of detection was not affected.

The overall performance of the RAZOR Anthrax System was extremely consistent during both validations. During the MD studies, 1370 of the 1372 samples tested at or above the AMDL yielded expected results (POD = 0.999, 95% CI extends from 0.9943 to >0.9999). During the ILV studies, 1090 of the 1094 samples tested at or above the AMDL yielded expected results (POD = 0.996, 95% CI extends from 0.9903 to 0.9989). This indicates that the RAZOR Anthrax System is a very reliable tool for detecting virulent *B. anthracis* in environmental samples generated by liquid or filter-based air collectors.

Conclusions

We recommend the RAZOR EX Anthrax Air Detection System for *Performance Tested Method* certification and submission for *Official Method of Analysis* collaborative study.

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Appendix 1
Pooling Scheme for Environmental Organisms

Pool 1.—Yersinia pestis (Colorado 92), Bacillus macroides, Burkholderia cepacia, Clostridium sardiniense, Pseudomonas aeruginosa, Mycobacterium smegmatis, Streptomyces coelicolor, Bacillus psychrosaccharolyticus, Streptococcus pneumonia, Synechocystis.

Pool 2.—Francisella tularensis Schu-4, Bacillus benzoevorans, Burkholderia gladioli, Clostridium perfringens, Fusobacterium nucleatum, Neisseria lactamica, Vibrio cholerae, Bacillus cohnii, Staphylococcus aureus, Acinetobacter lwoffii.

Pool 3.—Brucella melitensis, Coxiella burnetii, Bacillus megaterium, Burkholderia stabilis, Deinococcus radiodurans, Lactobacillus plantarum, Rhodobacter sphaeroides, Legionella pneumophila, Bacteroides fraginils, Stenotrophomonas maltophilia, Agrobacerium tumefaciens.

Pool 4.—Burkholderia pseudomallei, Clostridium botulinum, Bacillus horikoshii, Burkholderia plantarii, Delftia acidovorans, Moraxella nonliquefaciens, Riemerella anatipestifer, Escherichia coli, Shewanella oneidensis, Listeria monocytogenes.

Pool 5.—Aureobasidium pullulans, Alternaria alternata, Cladosporium cladosporioides, Cladosporium sphaerospermum, Epicoccum nigrum, Wallemia sebi.

Pool 6.—Aspergillus fumagatis, Eurotium amstelodami, Mucor recemosus, Paecilomyces variotii, Penicillum chrysogenum, Acanthamoeba castellanii, Naegleria fowleri.

Pool 7.—Mosquito (*Aedes albopictus*), Mosquito (Aedes aegypti), Dust mite (Dermatophagoides pteronyssinus), Cockroach (*Blatella germanica*), Flea (*Xenopsylla cheopis*), Fruit fly (*Drosophila melanogaster*), Housefly (*Musca domestica*), Tick (*Ixodes scapularis*).

Pool 8.—Herpes simplex virus, Vaccinia virus, Adenovirus, Coddling virus (Cyd-X, coddling moth virus), Lymentia virus (Gyp-chek, Gypsy moth virus), *Chryseobacterium indologenes.*

Test individually at 10X AMDL or the highest achievable concentration if DNA recovery is lower than 10X AMDL.—Rat (*Rattus norvegicus*, No. 9 in Table 24), Dog (*Canis familiaris*, No. 10 in Table 24), Human (*Homo sapien*, HeLa cell line, No. 11 in Table 24), Chicken (*Gallus gallus*, No. 12 in Table 24), Pine (*Pinus pinea*, No. 13 in Table 24), Corn (*Zea mays*, No. 14 in Table 24), Cotton (*Gossypium* sp., pollen, No. 15 in Table 24), Ricin plant (*Ricinus communis*, No. 16 in Table 24), Mouse (*Mus musculus*, No. 17 in Table 24), Cat (*Felis catus*, No. 18 in Table 24).