Multiplex Detection of Category A and B Biothreat Pathogens

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SUMMARY

OBJECTIVE: The study was designed as a proof of concept demonstration of the robustness available in Idaho Technology Inc.'s FilmArray® system. The automated sample processing, nested multiplex PCR, and results analysis features of the system were tested in the presence of organisms and various sample matrices that may be used to subvert, or confuse, molecular detection technologies.

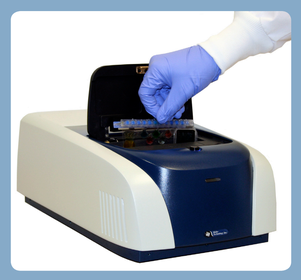
STUDY DESIGN: For sample matrix robustness testing, three soil types, four powders, and surface swabs were analyzed by the FilmArray system in the presence of a known NIAID category A or B pathogens. Specificity testing was performed using pooled samples of purified genomic template from phylogenetically related organisms, background organisms, and Biothreat spoofing agents. A co-spiking experiment was performed using three organisms at or near their limit of detection and one organism at 10^4-fold above its limit of detection.

RESULTS: The FilmArray was able to detect the B. anthracis, Y. pestis, and F. tularensis in the presence of soils and powders. Template manipulation area surface swab resulted in detection of Y. pestis and R. communis, both agents which were presumed to be contamination. Specificity testing was 100% against the exclusivity panel. B. anthracis, Y. pestis, and B. melitensis were all detected at 10^2 genomic equivalents per mL in the presence of S. aureus spiked at 10^6 genomic equivalents per mL (also detected), highlighting the dynamic range of nested multiplex PCR.

CONCLUSION: The FilmArray BioThreat pathogen detection system is capable of dealing with a variety of complex sample matrices with less than 5 minutes of hands-on time while providing highly reliable and actionable data.

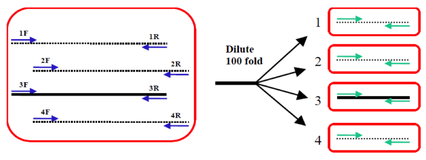
INTRODUCTION

The critical need for a robust molecular detection tool with a fast turn around time is well recognized by the Biodefense community. In addition, a system capable of detecting NIAID category A and B agents from diverse matrices is desired. In an effort to expedite the detection and identification of NIAID category A and B agents, Idaho Technology Inc. has developed a first generation BioThreat panel. The FilmArray BioThreat Panel was designed to detect the following pathogens: *B. anthracis*, *Y. pestis*, *F. tularensis*, *C. burnetii*, *Rickettsia* spp., *Brucella* spp., *Burkholderia* spp., Ebola, Marburg, equine encephalitis viruses (Eastern, Western, Venezuelan), Variola major, Orthopox, and the genes encoding Ricin toxin, Staphylococcal enterotoxin B, and Botulinum toxin.



As illustrated here, the FilmArray BioThreat Panel is a small footprint instrument that performs an integrated sample preparation processes then uses multiplex nested PCR (**Figure 2**) and LC Green®+ chemistry to automatically analyze a sample with minimal operator input. Melt analysis is used for detection which allows increased specificity in organism identification. This panel was used to test various spiked sample matrices (soil, swab, powder, and spoofing agent) for the presence of BioThreat agents. Additional specificity testing was performed with phylogenetically related species, background organisms, and BioThreat spoofing agents with results demonstrating high specificity.

FIGURE TWO: Schematic of Multiplex Nested PCR



A large volume multiplex PCR (shown here as 4-plex on the left side of figure) is run for a limited number of cycles (26). The reaction is diluted and distributed to individual small PCR reactions that contain primers (green) nested inside the primers (blue) of the first PCR reaction. A template amplified in the first reaction (by the #3 primers) is further amplified in only one of the second reactions.

FIGURE ONE: The FilmArray Instrument and Pouch

ITI has developed a lab-in-a-pouch system called "FilmArray". It is a medium-scale fluid manipulation system performed in a self-contained, disposable, thin-film plastic pouch. The FilmArray platform processes a single sample, from nucleic acid purification to result, in a fully automated fashion. These system characteristics are ideal for the multiplex testing of pathogens in various sample matrices.

The FilmArray Test System

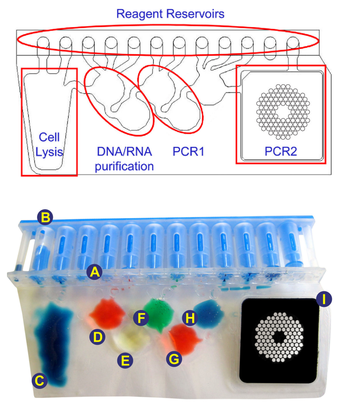
A FilmArray test is initiated by injecting rehydration solution and a sample into the FilmArray pouch and placing it in the FilmArray instrument. The user enters the sample and pouch type (using a barcode reader) into the software and initiates a run. Results are provided in about 1 hour.

The FilmArray Pouch

The pouch has a fitment (A) containing all needed freeze-dried reagents. The film portion of the pouch has stations for:

1. Cell lysis (C)
2. Magnetic-bead based nucleic acid purification (D & E)
3. First-stage multiplex PCR (F & G)
4. Array of 102, second-stage nested PCRs (I)

PCR primers are dried into the wells of the array and each primer set amplifies a unique product of the first-stage multiplex PCR. The second stage PCR product is detected in real-time using a fluorescent-double-stranded DNA binding dye, LCGreen+.



A. Fitment with freeze-dried reagents
B. Plungers- deliver reagents to blisters
C. Sample lysis and bead collection
D. Wash station
E. Magnetic bead collection blister
F. Elution Station
G. Multiplex Outer PCR blister
H. Dilution blister
I. Inner Nested PCR array

RESULTS AND DISCUSSION

Three soil types (sandy clay loam, sandy loam, and loamy sand) were spiked with 3000 cfu of *F. tularensis*. No inhibitory affects by the soils were observed. **Figure Three** shows the detection of *F. tularensis* in sandy clay loam soil.

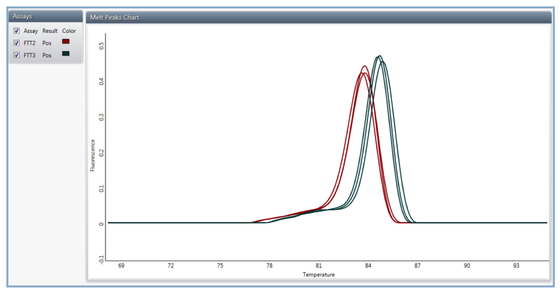
Four powders (baking powder, talcum powder, chocolate powder, and Dipel) were spiked with organism and tested by the FilmArray BioThreat Panel. The baking powder and chocolate powder were spiked with 3000 cfu of *F. tularensis*. The talcum powder and Dipel were spiked with 3200 cfu of *B. anthracis* (Sterne). No inhibitory affects by the powders were observed. **Figure Four** shows the detection of *B. anthracis* (Sterne) in Dipel powder. The *B. thuringiensis* Kurstaki spores did not inhibit detection of *B. anthracis* (Sterne).

An environmental surface swab was tested by the FilmArray BioThreat Panel as a proof of concept. A surface was spiked with *R. communis* and *Y. pestis* DNA then swabbed. The swab proof of concept resulted in amplification of the *R. communis* and *Y. pestis* assays. **Figure Five** shows the detection results for both *R. communis* and *Y. pestis* from the swabbed surface.

A high-low multiplex test of *B. anthracis* (Ames), *B. melitensis*, *Y. pestis*, and *S. aureus* demonstrated the Film Array's ability to detect multiple low concentration templates in the presence of a high copy template. The *B. anthracis*, *B. melitensis*, and *Y. pestis* were added at 100 genomic equivalents per mL. The *S. aureus* was added at 100,000 genomic equivalents per mL. The high concentration *S. aureus* template did not prevent the detection of the low copy templates in multiplex. **Figure Six** shows the assay melt profiles for each organism used during the high-low test.

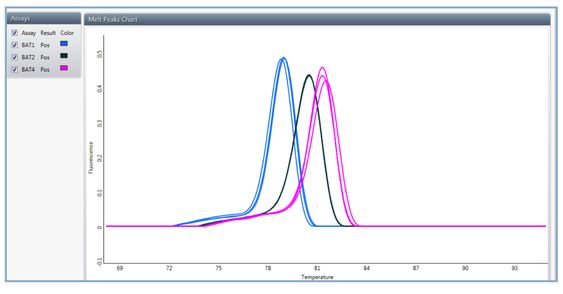
A 55 organism exclusivity panel containing FilmArray BioThreat Panel near neighbors was tested and demonstrated the BioThreat Panel assays have high specificity toward their intended organisms. **Table One** lists the organisms tested against the BioThreat Panel. Nucleic acid stock preparations of each organism were used at $5x10^8$ genomic equivalents per mL. A maximum of five nucleic acid preparations were pooled and tested by the FilmArray BioThreat Panel. One nucleic acid pool produced a *Brucella* species positive result. The *Brucella* species positive result is not thought to be a result of cross reactivity and is believed to be from *Brucella* species contamination of a nucleic acid preparation. Further investigation by testing each nucleic acid individually will determine the cause of the positive result. A Staphylococcal enterotoxin B positive result was obtained from another organism pool and is attributed to the *S. aureus* genomic template within the pool.

FIGURE THREE: Sandy Clay Loam Soil with *F. tularensis* Detection



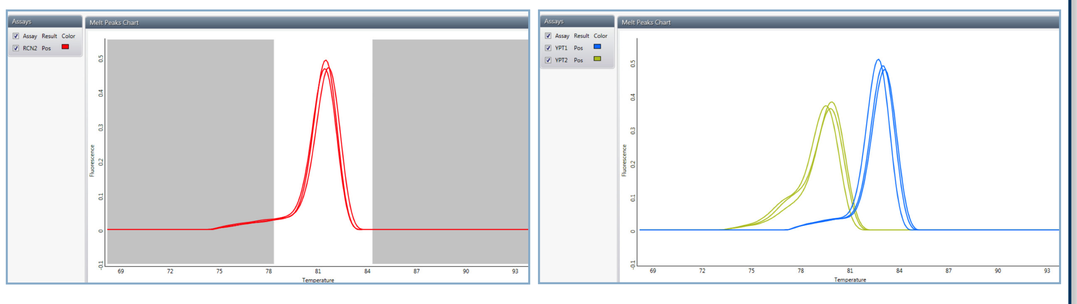
Detection of *F. tularensis* in sandy clay loam soil at 3000 cfu/sample. No other agents were detected. The melt profiles of the two assays targeting *F. tularensis* were identified.

FIGURE FOUR: Dipel Powder with *B. anthracis* (Sterne) Detection



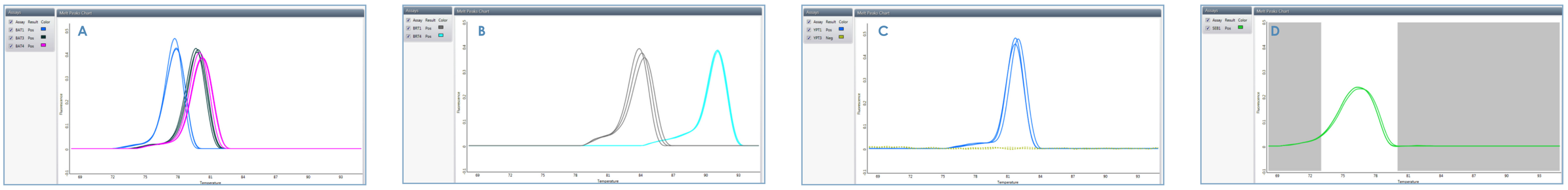
Detection of *B. anthracis* (Sterne) in Dipel powder at 3200 cfu/sample. No other agents were detected. The melt profiles for three of the assays targeting *B. anthracis* were identified. The first pXO1 (blue), second pXO1 (dark green), and chromosomal element (pink) assays produced positive melt profiles.

FIGURE FIVE: Surface Swab Proof of Concept



Detection of *R. communis* (left) and *Y. pestis* (right) from a surface swab. The *R. communis* assay (left, red) shows the melt profile and allowed melt profile range between the two shaded areas. Both *Y. pestis* assays (right, blue and yellow) detected the presence of template from the surface swab.

FIGURE SIX: High-low Multiplex PCR Detection



High-low multiplex PCR of *B. anthracis*, *B. melitensis*, *Y. pestis* and *S. aureus*. *B. anthracis* (A), *B. melitensis* (B), *Y. pestis* (C) were detected at 100 genomic equivalents per mL. *S. aureus* (D) was detected at 100,000 genomic equivalents per mL. One of two assays for *Y. pestis* failed to amplify (shown by dashed line) because the 100 genomic equivalents per mL concentration is known to be below the assay's limit of detection.

TABLE ONE: Exclusivity Panel Organisms Tested Against the BioThreat Panel

Organism	Targets Detectable By FilmArray	FilmArray Assay Result
Acinetobacter baumanni		No detection
Enterobacter aerogenes		No detection
Enterobacter agglomerans		No detection
Yersinia pseudotuberculosis (YERS091)		No detection
Yersinia pseudotuberculosis (YERS092)		No detection
Bacillus cereus (BACI015)		No detection
Bacillus cereus (BACI016)		No detection
Bacillus cereus (BACI227)		No detection
Bacillus cereus (BAC I228)		No detection
Bacillus cereus (BACI290)		No detection
Bacillus megaterium (BACI026)		pXO1 positive
Bacillus mycoides (BACI028)		pXO1 positive
Bacillus subtilis var niger (BACI051)		pXO1 positive
Bacillus cereus G9241	pXO1+	pXO1 positive
Bacillus mycoides (BACI088)		pXO1 positive
Bacillus subtilis var niger (BACI034)		No detection
Bacillus thuringieinsis israelensis		No detection
Bacillus thuringieinsis (BACI036)		No detection
Bacillus thuringieinsis (BACI172)		No detection
Bacillus thuringiensis (BACI265)		No detection
Bacillus thuringiensis (Kurstaki)		No detection
Bacillus thuringiensis (Al Hakam)		No detection
Bacillus thuringiensis (97-27)		No detection
Francisella philomiragia (FRAN002)		No detection
Francisella philomiragia (FRAN017)		No detection
Haemophilius influenzae		No detection
Klebsiella pneumoniae		No detection
Moraxella cattaharalis		No detection
Pasteurella multocida		No detection
Proteus vulgaris		No detection
Providencia stuartii		No detection
Pseudomonas aeruginosa		No detection
Ralstonia pickettii		No detection
Serratia marcescens		BRT1 positive
Shigella flexneri		BRT1 positive
Shigella sonnei		BRT1 positive
Yersinia kirstensenii (Y225)		BRT1 positive
Yersinia aldovae		BRT1 positive
Yersinia frederiksenii		No detection
Yersinia enterocolitica (YERS014)		No detection
Yersinia enterocolitica (2516-87)		No detection
Yersinia enterocolitica (Y231)		No detection
Yersinia frederiksenii (670-83)		No detection
Yersinia pseudotuberculosis (YERS008)		No detection
Yersinia pseudotuberculosis (IP32953)		No detection
Yersinia pseudotuberculosis (YPIII)		No detection
Yersinia pseudotuberculosis (Pa3606)		No detection
Yersinia pseudotuberculosis (MD67)		No detection
Listeria monocytogenes		SEB1 positive
Staphylococcus aureus	entB	SEB1 positive
Staphylococcus hominis		SEB1 positive
Streptococcus pyogenes		SEB1 positive
Streptococcus pneumoniae		SEB1 positive
Bacillus cereus (03BB102)	pXO1+, pXO2+	pXO1 and pXO2 positive
Francisella novicida (ATCC 15482)		FTT2 and FTT3 positive

Exclusivity panel test results against the FilmArray BioThreat Panel. A maximum of five genomic templates at $5x10^8$ genomic equivalents per mL were pooled (pools shown by shading) and tested against the BioThreat Panel.

CONCLUSION AND FUTURE STUDIES

The FilmArray BioThreat Panel is a robust molecular detection tool that will improve public safety by providing fast, sensitive, and specific BioThreat agent detection from various sample matrices. Future studies will involve inclusivity panel testing with live agents and the addition of more sample matrices and spoofing agents.

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