

# Autonomous Detection of Aerosolized Biological Agents by Multiplexed Immunoassay with Polymerase Chain Reaction Confirmation

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The autonomous pathogen detection system (APDS) is an automated, podium-sized instrument that continuously monitors the air for biological threat agents (bacteria, viruses, and toxins). The system has been developed to warn of a biological attack in critical or high-traffic facilities and at special events. The APDS performs continuous aerosol collection, sample preparation, and detection using multiplexed immunoassay followed by confirmatory PCR using real-time TaqMan assays. We have integrated completely reusable flow-through devices that perform DNA extraction and PCR amplification. The fully integrated system was challenged with aerosolized *Bacillus anthracis*, *Yersinia pestis*, *Bacillus globigii*, and botulinum toxoid. By coupling highly selective antibody- and DNA-based assays, the probability of an APDS reporting a false positive is extremely low.

Aerosol monitoring systems that test for the presence of biological agents are in use throughout the United States.<sup>1</sup> Their purpose is to detect a biological agent release so that early public health action may be taken. The impact of a biological agent attack can be reduced if the appropriate responses are executed with minimal delay.<sup>1</sup> For example, at the 2002 Winter Olympics in Salt Lake City, aerosol samples from dry filter collectors were periodically transported to a centralized field laboratory where they were screened for the presence of pathogen specific nucleic acid sequences.

The autonomous pathogen detection system (APDS) is a next-generation biological agent detector that was developed to realize in situ sample collection, analysis, and result reporting. The benefits of this approach include reduced operational costs and faster data reporting while maintaining a low false positive rate. McBride et al.<sup>2</sup> described an earlier version of APDS that performed aerosol collection, sample preparation, and multiplexed

immunoassay detection; detection of live *Bacillus anthracis* and *Yersinia pestis* was demonstrated during laboratory chamber testing of the device. Since this report, DNA detection capability has been added to the APDS to further reduce the likelihood of false positives; now, a suspect immunoassay result initiates a confirmatory real-time flow-through polymerase chain reaction (PCR) assay. Belgrader et al.<sup>3</sup> have described the real-time flow-through PCR module employed in the APDS. As certain sample matrixes can interfere with the PCR assay, a DNA extraction device was also added.

In its current configuration, the APDS (Figure 1) operates continuously for 7 days; an immunoassay result is reported every hour, and a control PCR reaction is executed twice daily. A suspicious immunoassay result is identified using a signal analysis algorithm, which triggers the corresponding confirmatory PCR assay. Together, these technical advances culminated in laboratory chamber tests during which the APDS was challenged with individual aerosol releases of *B. anthracis*, *Y. pestis*, *Bacillus globigii* (a *B. anthracis* simulant), and botulinum toxoid (inactivated botulinum toxin). The APDS data presented herein demonstrate for the first time completely autonomous biological monitoring using aerosol sample collection, multiplexed immunoassay analysis, DNA extraction, and confirmatory PCR.

## EXPERIMENTAL SECTION

The previous version of the APDS, which incorporated an aerosol collector, a sample preparation module, and multiplexed immunoassay flow cytometric detection, has been described in previous papers.<sup>2,4</sup> The current APDS also employed a Luminex 100 flow cytometer (Luminex Corp., Austin, TX) fitted with a sheath delivery system (Luminex). Antibodies from Tetracore (Gaithersburg, MD) were conjugated to Luminex microspheres (Luminex) according to the manufacturer's protocols. The sample preparation module was a modified sequential injection analysis system (Global FIA, Fox Island, WA) fitted with two syringe pumps (1 mL, XP3000, Cervo, Sunnyvale, CA) and four multipo-

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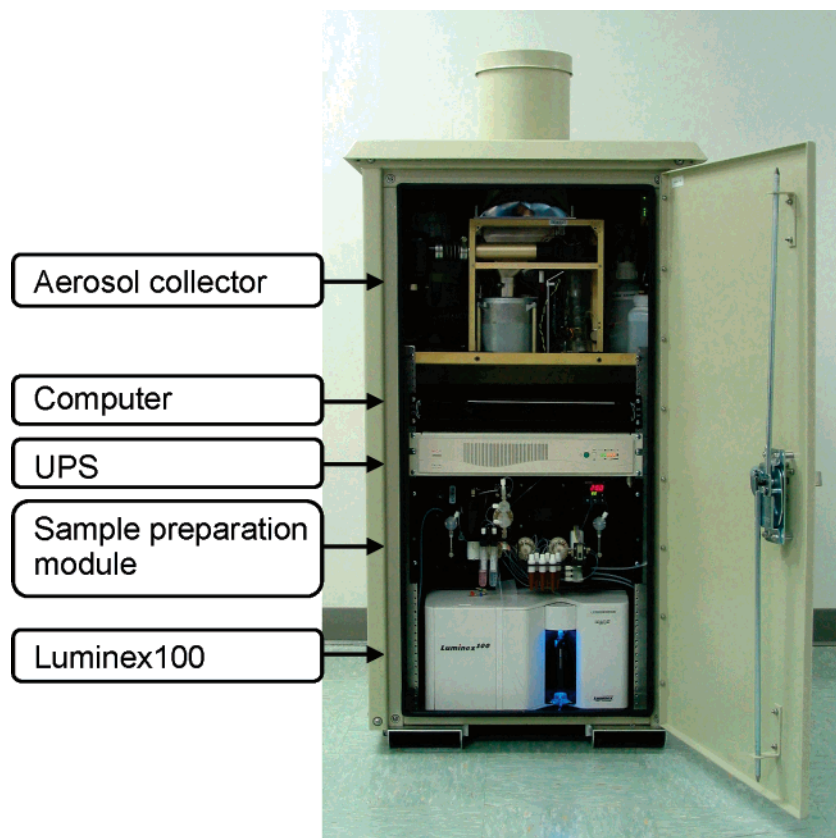
<sup>‡</sup> United States Army Dugway Proving Grounds.

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**Figure 1.** Autonomous pathogen detection system, including an aerosol collector, a laptop computer, an uninterruptible power supply (UPS), a sample preparation module configured for multiplexed immunoassay, DNA extraction and confirmatory flow-through PCR, and a Luminex100 flow cytometer. The APDS height, width, and depth are 1.44, 0.72, and 0.88 m, respectively. The cabinet weighs  $\sim 170$  kg. Fluid reservoirs, sized for 1 week of continuous operation, are located at the rear of the cabinet.

sition selection Cheminert valves ( $2 \times 10$  and  $2 \times 14$  port, VICI, Houston, TX). PFA tubing (0.8-mm i.d., 1.6-mm o.d., Cole-Parmer, Vernon Hills, IL) was used throughout the fluidics manifold. Flangeless 1/4–28 and 10–32 nuts and ferrules (VICI) provided tubing connections throughout the manifold.

**System Control.** APDS components, including the aerosol collector, sample preparation module, and Luminex 100 were controlled by a laptop computer running a graphical user interface written in Labview Version 6.1 (National Instruments, Austin, TX). Communication between the Labview graphical user interface and the Luminex 100 was achieved using the software program Luminex LXR Library Version 2.6.9 (Luminex). Both serial cables and data acquisition cards (DAQ1200, National Instruments) were used for hardware communications and data transfer throughout the system.

**Immunoassay Signal Analysis.** Baseline median fluorescence intensity (MFI) values for 11 agents and 4 controls were established from samples ( $n = 24$ ) of blank chamber air that were collected and analyzed by the APDS at Dugway. For each microsphere class, the mean baseline value and standard deviation were calculated and entered into the system. During autonomous operation, the immunoassay results for a given sample were first divided by their respective baseline MFI value to create a normalized signal. The highest agent normalized signal was then divided by the second highest normalized signal (including the remaining 10 agents and the negative control) to yield a relative signal, which accounted for baseline drift and nonspecific binding.

The relative signal was checked against an established threshold value calculated from titration data collected previously in our laboratory. The system also checked that  $>1500$  classified microspheres were counted and that control MFI values were valid (baseline  $\text{MFI} \pm 3\sigma$ ). If a relative signal for a given agent exceeded its threshold, the corresponding confirmatory PCR reaction was triggered.

**Automated DNA Extraction.** Christel et al.<sup>5</sup> have described a micromachined silica pillar device similar to that used within the APDS. The chip was fabricated within our laboratory using deep reactive ion etching to yield a square pillar bed with a void volume of  $3 \mu\text{L}$  and a total surface area of  $\sim 2 \text{ cm}^2$ . The pillar diameter, length, and spacing were 20, 200, and  $20 \mu\text{m}$ , respectively. A platinum thin-film resistor was deposited on the backside of the chip to enable heating of the device during DNA elution.

Aerosol collector sample ( $100 \mu\text{L}$  of a total  $4 \text{ mL}$ ) and chaotropic solution ( $100 \mu\text{L}$ ,  $2 \text{ M}$  guanidine thiocyanate,  $3.3 \text{ mM}$  Tris,  $0.33 \text{ mM}$  EDTA, pH 6.5, Teknova, Hollister, CA) were mixed by the automated fluidics system and then pumped through the silica bed at  $1 \mu\text{L/s}$  to extract the DNA. The bed was washed with ethanol ( $300 \mu\text{L}$  of  $70\% \text{ v/v}$  and then  $100 \mu\text{L}$  of  $95\% \text{ v/v}$ ) to remove potential PCR interferences. The DNA was eluted in Tris/EDTA buffer ( $10 \mu\text{L}$ ,  $10 \text{ mM}$  Tris,  $1 \text{ mM}$  EDTA, pH 8.0, Acros, Morris Plains, NJ) with heating to  $80^\circ\text{C}$  and then introduced as sample ( $5 \mu\text{L}$ ) to the flow-through PCR module. After each

(5) Christel, L. A.; Petersen, K.; McMillan, W.; Northrup, M. A. *J. Biochem. Eng.* **1999**, *12*, 22–27.

extraction, the chip was decontaminated with sodium hypochlorite (100  $\mu$ L, 1.25% m/v) and then rinsed with deionized water (2 mL). The extraction chip was filled with deionized water when not in use.

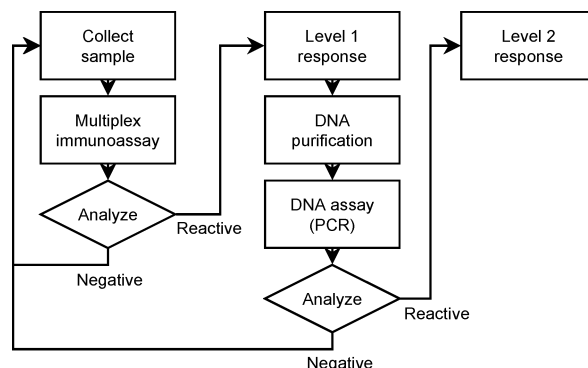
**Automated Flow-Through PCR.** The single-chamber flow-through PCR module has been described previously.<sup>3</sup> When triggered by a reactive immunoassay result, the automated fluidics module assembled the corresponding confirmatory PCR reaction (25  $\mu$ L) from enzyme master mix solution (15  $\mu$ L, Accuprime Supermix I, MgCl<sub>2</sub> 4 mM, Invitrogen, Carlsbad, CA), a mixed solution of DNA primer pairs, TaqMan probe specific for the agent in question (5  $\mu$ L, 200 nM each, probe was FAM at 5' end and quenched by Black Hole Quenchers at 3' end, Biosearch Technologies, Novato CA), and sample (5  $\mu$ L). The resultant reaction mixture was positioned in the tubing of the flow-through PCR heater and subjected to a thermal cycling protocol of 95 °C for 120 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s. A fluorescence measurement (480-nm excitation, 520-nm emission) was made during each cycle, after completion of the 60 °C hold. After thermal cycling had finished, the reactor tubing was automatically cleaned with sodium hypochlorite (500  $\mu$ L, 1.25% m/v) followed by a water rinse (4 mL). This cleaning procedure prevented any carryover of amplified DNA and regenerated the PCR module for the next assay. All PCR reagents were stored at ambient temperature within individual reservoirs on the fluidics module. The flow-through PCR tubing was filled with deionized water when not in use.

**Biological Agents.** Certified killed ( $\gamma$ -irradiated) *B. anthracis* spores (Ames strain) and *Y. pestis* vegetative cells (India 195/p strain) were from Dugway Proving Grounds (Dugway, UT). Viable *B. globigii* spores were also from Dugway Proving Grounds. Botulinum toxoid (an inactivated form of botulinum toxin) was from Tetracore.

**Aerosol Chamber Test Procedure.** The APDS was tested in the Aerosol Simulant Exposure Chamber at the West Desert Test Center, United States Army Dugway Proving Grounds. The chamber (5  $\times$  5  $\times$  3 m) was equipped with a Babbington aerosol generator (referred to here as the nebulizer) that aerosolized liquid samples at variable flow rates (0.2–5 mL/min). The subsequent aerosol was thoroughly mixed with chamber air by a bank of fans; each release lasted for 50 min and coincided with the collection period. The air turnover rate within the chamber was between 1400 and 14000 L/min. The aerosol collector was removed from the chassis and positioned in the center of the chamber while other APDS components remained outside the chamber; fluid and communication connections were made via a sealed port. The liquid sample was automatically pumped from the aerosol collector to the fluidics module hourly.

Bioaerosol reference measurements were made. An aerodynamic particle sizer (APS) provided real-time particle size measurements (0.5–30  $\mu$ m) prior to and during each release. All-glass impinger air samplers (AGI-30s, Ace Glass Co. Vineland, NJ) and slit-to-agar biological air samplers (New Brunswick Scientific, Edison, NJ) were also utilized. For disseminations, antigen slurry (100 mL, prepared in deionized water) was loaded into the nebulizer. A target APS count was maintained for the duration of each release by cycling on and off the nebulizer's peristaltic pump.

## Scheme 1. APDS Flow Diagram Showing the Cycle of Sample Collection, Multiplexed Immunoassay, DNA Extraction, and Confirmatory PCR Analysis<sup>a</sup>



<sup>a</sup> Aerosol collection and sample analysis occur concurrently. Multiplexed immunoassay and confirmatory PCR each have analysis times of 30–60 min. Assay results and system status are continuously transmitted through a secure wireless network.

**Decontamination.** After each agent release, the chamber was purged with clean air, until the APS indicated that the particle counts had returned to baseline. The nebulizer, AGIs, and slit samplers (when used) were retrieved from the chamber. Both the chamber floor and aerosol collector were decontaminated with sodium hypochlorite (0.6% m/v) and then rinsed with water. The water from the final aerosol collector rinse was collected and then analyzed by real-time PCR to verify this component was clean before we proceeded with the next test.

**Safety Considerations.** Personal protective equipment (gloves, lab coat, goggles, positive air pressure respirators) was worn. Consumables (filtration plates, pipet tips, tubes, etc.) and aqueous waste were collected in biohazard bags and autoclaved. The instrumentation was decontaminated with sodium hypochlorite solution (1.25% m/v) after exposure to biological agents.

## RESULTS AND DISCUSSION

**Autonomous Pathogen Detection System.** A process flow diagram of the APDS with PCR confirmation is shown in Scheme 1. The aerosol collector samples up to 3300 L of air/min, and airborne particles are trapped in a small volume of water (4 mL) within a wetted wall cyclone that is analyzed periodically (hourly in this case). The multiplex immunoassay is the primary screen, and the instrument operates in the first loop (Scheme 1, left-hand side) for most of the time. A reactive immunoassay result causes a level 1 response that represents actions taken by both the instrument (e.g., paging an expert with a request for data review) and the external systems (e.g., security cameras checked). If the reactive agent contains DNA, then PCR confirmation is initiated. A reactive PCR assay result causes a more extensive level 2 response. The use of two highly specific biological assays that each rely on fundamentally different molecular interactions for detection boosts confidence in the results, which in turn enables decisive (level 2) responses to begin before samples could even be transported to a field laboratory for analysis.

**Multiplexed Microsphere Immunoassay.** The current multiplexed microsphere immunoassay is an extension of those



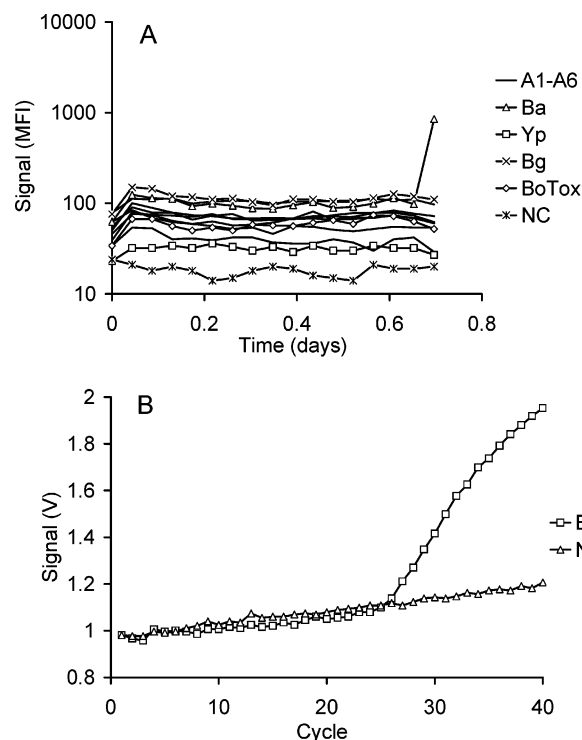
described previously.<sup>2,4,6</sup> In short, antigens form a “sandwich” with antibody-coated microspheres (5.6  $\mu\text{m}$ ) and detector antibodies labeled with streptavidin phycoerythrin; the resulting suspension is read with a Luminex 100 flow cytometer. The microspheres are color-coded to identify the antigen type; the fluorescence intensity of bound streptavidin phycoerythrin corresponds to antigen concentration. Up to 100 different microsphere classes can be distinguished in one sample. The assay panel used in this study tested for 11 biological agents simultaneously, including *B. anthracis*, *Y. pestis*, *B. globigii* (a *B. anthracis* simulant), and botulinum toxoid (inactivated botulinum toxin). In addition to the 11 agent microsphere types, 4 control microsphere classes were included in each assay: negative control, instrument control, fluorescent control, and antibody control.<sup>2,4</sup> These internal assay controls provide important diagnostic information during extended autonomous operation.

**PCR Assays.** The APDS uses DNA assays based on real-time TaqMan PCR, where cleavage of fluorescent resonance energy transfer quenched probes during amplification results in an increased fluorescence signal.<sup>7,8</sup> The assay is performed in a flow-through PCR module described by Belgrader et al.<sup>3</sup> We took this module and made the PCR analysis fully automated; i.e., after a suspicious immunoassay result, the system selected the corresponding PCR reagents, mixed them with sample, performed the PCR analysis, and then decontaminated fluid lines and components in preparation for the next analysis. During routine operation, the system can accommodate parallel execution of both the immunoassay and PCR.

**Nucleic Acid Extraction.** Certain environmental sample matrixes contain species that can inhibit PCR and prevent DNA amplification from occurring. To purify nucleic acids from inhibitors that could be collected from the environment, the APDS performs sample preparation by microchip-based solid-phase extraction prior to PCR. In this process, DNA is captured on microfabricated silica pillars, while the sample matrix including potential interferences pass through to waste. The immobilized DNA is washed, eluted, and then introduced into the PCR reaction. During our testing at Dugway, the APDS collected samples of relatively clean chamber air that did not contain PCR inhibitors. The nucleic acid extraction module, however, was enabled during chamber tests to evaluate its function as an integral component of the APDS. Input and elution volumes used by the extraction module were 100 and 10  $\mu\text{L}$ , respectively. For the extraction module alone, input DNA concentrations ranging from  $10^3$  to  $10^7$  copies/mL realized concentrations factors of  $\sim 1$ –2 or recoveries of  $\sim 10$ –20% (data not shown). As PCR is at least 50-fold more sensitive than our multiplexed immunoassay, this extraction performance was adequate for the confirmatory PCR assay. Automated microchip DNA purification from PCR inhibitory environmental aerosol samples will be published in a separate report.

#### Chamber Testing of the APDS with Aerosolized Biological Agents.

We conducted releases of *B. anthracis* spores, *Y. pestis*



**Figure 2.** Autonomous pathogen detection system chamber test results that demonstrated (A) multiplexed immunoassay identification followed by (B) DNA extraction with PCR confirmation of an individual aerosol release of *B. anthracis*. Immunoassay signal designations are A1–A6 (agents 1–6), Ba (*B. anthracis*), Yp (*Y. pestis*), Bg (*B. globigii*), BoTox (botulinum toxoid), and NC (negative control). MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release. A negative control (NC) for PCR confirmation is shown for reference; this was obtained prior to the *B. anthracis* release (A) using deionized water as the sample.

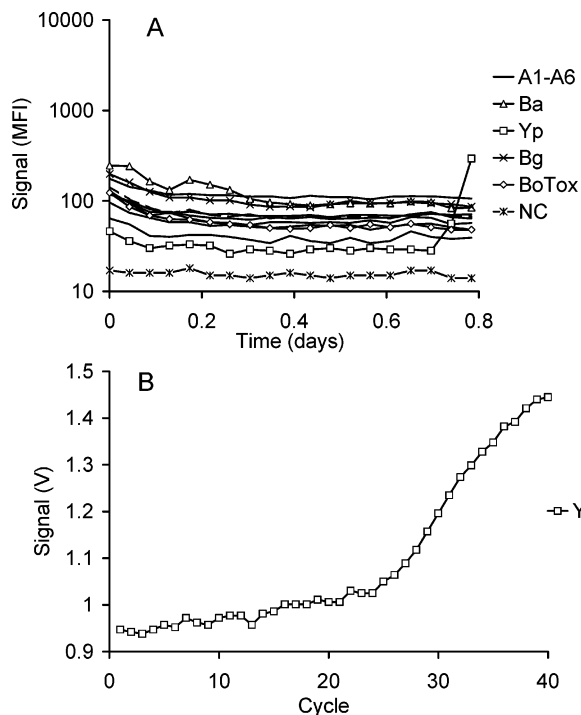
vegetative cells, *B. globigii* spores, and botulinum toxoid. Each test point consisted of collecting the disseminated aerosol, preparing and reading the multiplexed immunoassay, identifying a reactive immunoassay result with a signal analysis algorithm, DNA extraction, real-time flow-through PCR, and observing a cycle threshold, all in an unattended continuous mode of operation. Confirmatory PCR was not performed for the toxoid release; being a protein, the presence of target DNA was not guaranteed. For these experiments, the system ran autonomously overnight and collected hourly aerosol samples of clean chamber air (blanks) that were analyzed by multiplexed immunoassay; an agent was released the following morning without interrupting the APDS. After the system had completed its analysis of the release, it was stopped and then decontaminated.

The multiplexed immunoassay results for *B. anthracis* are shown in Figure 2, where the MFI values of the 11 agents and the negative control are plotted versus time. The MFI values for all microsphere classes remained within  $3\sigma$  of their mean baseline value until a sudden increase of the *B. anthracis* signal that coincided with the aerosolization of *B. anthracis* spores. Note that the MFI values of the other microsphere classes remained within  $3\sigma$  of their mean baseline in the presence of *B. anthracis*, indicating our multiplexed immunoassays are highly specific. The subsequent PCR assay was triggered and provided clear confirma-

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(7) Heid, C. A.; Stevens, J.; Livak, K. J.; Williams, P. M. *Genome Res.* **1996**, *6*, 986–994.

(8) Nasarabadi, S.; Milanovich, F.; Richards, J.; Belgrader, P. *BioTechniques* **1999**, *27*, 1116–1118.



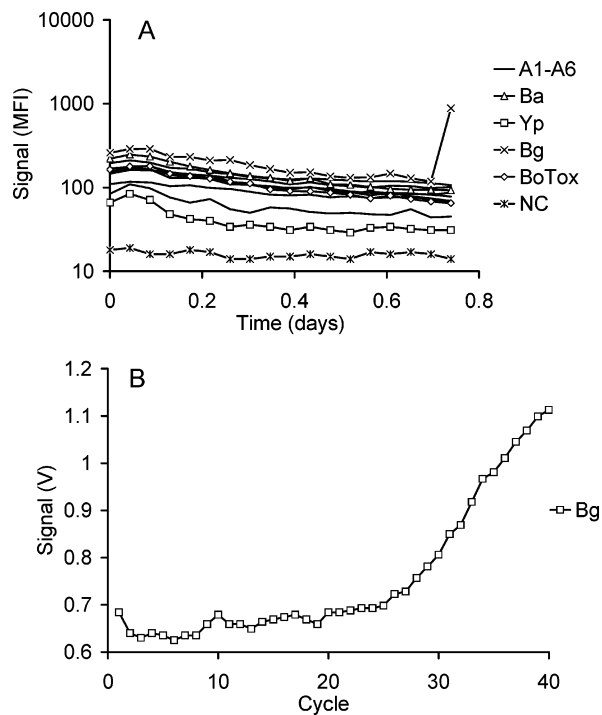
**Figure 3.** Autonomous pathogen detection system chamber test results that demonstrated (A) multiplexed immunoassay identification followed by (B) DNA extraction with PCR confirmation of an individual aerosol release of *Y. pestis*. Immunoassay signal designations are as given in the caption to Figure 2. MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release.

tion of the reactive immunoassay result. We used deionized water as negative control for the PCR confirmation assay prior to the *B. anthracis* release, which verified the system was clean prior to the release.

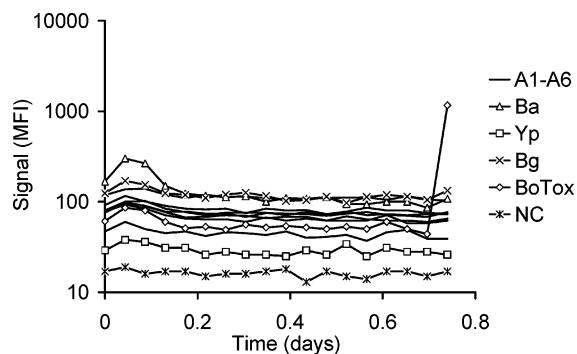
The initial *Y. pestis* release (Figure 3) generated an MFI value that was not detected by the signal analysis algorithm in use at the time and as a result, confirmatory PCR was not initiated. The *Y. pestis* aerosol concentration was increased during the next collection cycle; the increased MFI value in the subsequent immunoassay was detected by the algorithm and triggered confirmatory PCR with an unequivocal result. We have since changed the signal analysis algorithm used by the APDS to a moving window average based upon partial least-squares regression.

The APDS data from a *B. globigii* release is also shown in Figure 4. We used viable *B. globigii* spores to estimate the number of colony forming units present in the air during this dissemination. Slit samplers collected airborne particles directly onto culture plates that were subsequently incubated and then analyzed. Using this method, we calculated that the average agent-containing particle per liter of air value was 49. The results from this experiment demonstrate that the APDS is capable of achieving the sensitivity required by many real-world environmental monitoring scenarios.

Finally, detection of aerosolized botulinum toxoid is shown (Figure 5), which highlighted the versatility of the multiplexed immunoassay platform in its ability to identify the presence of pathogenic proteins, in addition to bacteria.



**Figure 4.** Autonomous pathogen detection system chamber test results that demonstrated (A) multiplexed immunoassay identification followed by (B) DNA extraction with PCR confirmation of an individual aerosol release of *B. globigii*. Immunoassay signal designations are as given in the caption to Figure 2. MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release.



**Figure 5.** Autonomous pathogen detection system chamber test results that demonstrated multiplexed immunoassay identification of an individual aerosol release of botulinum toxoid. Immunoassay signal designations are as given in the caption to Figure 2. MFI is the median fluorescence intensity shown for 11 agents and the negative control. Chamber background aerosol samples were collected and analyzed by the system prior to each release.

## CONCLUSIONS

We have demonstrated, for the first time, completely autonomous detection of aerosolized *B. anthracis* and *Y. pestis* by multiplexed immunoassay with confirmatory PCR. With the addition of DNA detection capability, the probability of an APDS reporting a false positive is extremely low. We also realized the detection of a botulinum toxoid aerosol that highlighted the system's ability to respond to protein toxins, in addition to bacteria. We have since fabricated multiple APDS units, equipped with the functionality described and demonstrated herein, that are under-

going extensive environmental testing in real-world environments, including subways and airports. The nucleic acid extraction module has undergone further characterization; the automated purification of DNA from environmental aerosol samples known to inhibit the PCR will be described in a separate report. The next version of the APDS, to be field tested this year, will incorporate multiplexed PCR with Luminex microsphere-based hybridization detection.

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