

### BEYOND CLINICAL DIAGNOSTICS

#### **Integrating detection and identification of biothreat agents.**

**Providing rapid results to ensure effective protection response is a primary goal of next-generation biodefense testing systems.** *By Colette A. Côté*

The public perception of the risks and dangers of potentially imminent biothreats has changed considerably since certain events occurred in the 1990s and earlier this decade. The 1995 sarin attack in the Tokyo subway system that killed 12 people and injured more than 5000, and the 2001 anthrax scare at U.S. postal offices that killed five and infected 15 made major headlines and served as a wake-up call. However, the 2004 ricin attacks in a U.S. Senate office building received far less attention. While the reasons for this discrepancy are varied, the fact remains that since public facilities and infrastructure, including the food and water supplies, are under constant threat from natural, accidental, and, more disturbingly, deliberate contamination, they require continuous monitoring.



The government, military, and private sectors in the United States have invested significantly into developing and implementing biowarfare-monitoring systems (e.g., the Laboratory Response Network and BioWatch). But such systems have come at the expense of lax food and environmental safety monitoring. The public has certainly been affected by the frequency and severity of the daily impact of food- and waterborne pathogens.

For example, in 2007, salmonella detected in spinach and peanut butter, and botulinum in chili and pet food led to significant recalls. Recently, *E. coli* fears prompted the recall of more than 21 million pounds of ground beef (the second-largest in U.S. history) and forced a meat company to close after 67 years in business. A 2006 recall involved spinach tainted with *E. coli* that killed three people and infected 200. CDC estimates that approximately 76 million cases of foodborne illnesses occur each year in the United States.<sup>1</sup> In addition, upwards of 2 million people become ill each year as a result of drinking contaminated water.<sup>2</sup>

Despite the myriad of technologies for food and water safety monitoring and pathogen detection, the U.S. Department of Agriculture (Washington, DC) and FDA (Rockville, MD) have used them haphazardly.<sup>3,4</sup> While recent incidents have garnered the public's desire for increased surveillance of the food supply, one lingering perception in the food industry has been that consumers should address many safety issues with proper handling techniques. This perception has reaffirmed the decisions by government entities to limit developing food and water surveillance technologies, and focus greater attention and resources on monitoring aerosol biothreats. Indeed, the release of bioterrorism agents as aerosols not only poses a tremendous risk to the entire population but also could overwhelm the ability of local, state, and federal agencies to respond. Such risks underscore further the need for rapid and efficient pathogen detection and identification systems.

This article discusses recent advances in developing comprehensive monitoring systems that can detect in real time and identify conclusively a biothreat from a broad range of pathogens and toxins. Such advances have been addressing the need to monitor and protect public health and safety from an ever-growing list of homeland security biothreats.

#### **Existing Technologies**

Although a multitude of technologies are available to identify pathogens, many are elaborate, time-consuming, or technically challenging. For example, traditional microbial culturing techniques can take several days or even weeks for analysis, and are better suited for secondary confirmation. More-rapid technologies that have been successfully developed include fluorescence imaging and other laser-based optic approaches that label and identify pathogenic organisms, e.g., the MicroPro by Advanced Analytical (Ames, IA); spectroscopy- and spectrometry-based systems for detecting biological and chemical agents, e.g., aerosol MALDI mass spectrometry, mobile infrared spectroscopy, and surface-enhanced Raman spectroscopy; and high-density protein microarrays for extensive proteome screening of pathogens, e.g., the ProtoArray by Invitrogen (Carlsbad, CA).

Next-generation immunoassays have made great strides since the days of traditional enzyme-linked immunosorbent assays. While such technologies, including microbead, fiber optic, piezoelectric, amperometric, and nanowire immunosensors are still being developed, they show great promise as applications for rapid, sensitive, high-throughput, and multiplexed pathogen detection.<sup>5-8</sup>

However, the current cost of equipment and reagents, overall assay complexity, and the need for greater technical expertise may limit first responders' abilities to use broadly such technologies in biothreat situations.

Real-time polymerase chain reaction (PCR)-based technologies, such as immuno-PCR techniques that combine the specificity of immunoassays with the amplification and quantification of real-time PCR, have emerged as a leading technology for rapid pathogen identification due to their speed and high degree of sensitivity and specificity.<sup>9-11</sup> However, the drawbacks of PCR have limited its potential as a first-responder technology. Such drawbacks include the high cost of equipment, training, and reagents; the high degree of false positives or negatives, which is a direct result of its ultrasensitivity; and the requirement of having some knowledge of the pathogen subgroup or subtype.

Other drawbacks are the frequent mutation rate that may affect priming sites (e.g., mispriming) and the fact that the DNA or RNA level present is not a direct indicator of function or pathogenicity.

Similarly, PCR-based assays for detecting agents such as dianthin and ricin have been limited to quasipure samples and are unlikely to detect such agents in their purest and most dangerous forms because of the lack of nucleic acids available for detection. Nonetheless, a number of rapid PCR-based systems have become the acceptable standard for assessing biothreat agents, given the need to identify quickly and efficiently their risk rather than their pathogenicity. But directly measuring pathogenicity is limited by the assays' ability to detect only one biothreat agent or a limited number of agents among many that may harm a population.

Advances in nucleic acid detection chemistries, technologies, and hardware have significantly shortened the analysis time from hours to about 30 minutes. The forefront technologies include handheld devices such as the Razor by Idaho Technologies (Salt Lake City, UT), the Bio-Seeq Plus by Smiths Detection (Edgewood, MD), and the GeneXpert real-time PCR system by Cepheid (Sunnyvale, CA). These PCR systems have been praised for their sensitivity and preformatted all-in-one cartridge or pouch-based systems that can assay a multitude of samples simultaneously.

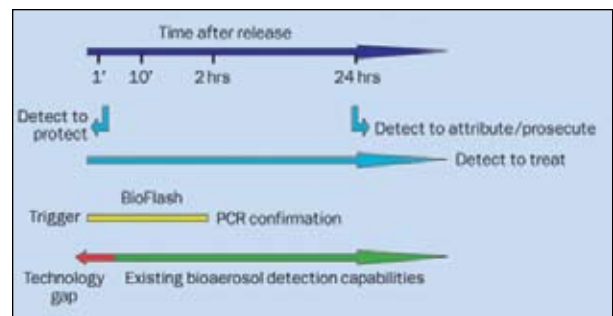


Figure 1. Threat detection turnaround time.

While the 30-minute time frame for results meets the Detect to Treat criteria, it falls short of the Detect to Protect window requiring results within 5 minutes or less (see Figure 1). Even though such existing technologies are suitable for food safety or general environmental monitoring, they are not acceptable for bioterror threats or other imminent environmental risks in which the speed of detection and identification is critical to lessen the immediate risk. There has been a recent surge in developing technologies that bridge the gap between the Detect to Protect and Detect to Treat time criteria.

## Bridging the Gap

The critical step for these biothreat monitoring technologies is integrating the detection and identification processes into a reliable, easy-to-use, and portable system. Such a system should operate as a constant sample collector or detector, and utilize a real-time trigger to prompt the identification system. But for many technologies, liquid-sample handling and complex matrix collection and preparation times have limited any reduction in the detection and identification times. This limitation has led to a greater emphasis on further developing comprehensive aerosol monitoring systems.

In 2003, the U.S. Department of Homeland Security established the BioWatch program. But this program was initially limited by the daily collection of airborne samples on filters that were sent to state and local public health laboratories for analysis.<sup>12</sup> While effective, the system was too slow and cumbersome to respond to threats in real-time. Lawrence Livermore National Laboratory (Livermore, CA) is developing several next-generation monitoring systems that have made significant advances in reducing the time between detection and identification. Such systems minimize end-user handling of samples and the use of consumables (thereby becoming more cost-effective), and also increase portability.<sup>12</sup>

For example, the Bioaerosol mass spectrometry system uses laser-induced fluorescence to obtain mass spectral signatures for identifying pathogenic bioaerosols. The Biobriefcase based on the MicroChemLab platform by Sandia National Laboratory (Albuquerque) utilizes microfluidic, chip-based modules for multiplex pathogen detection. ICx Technologies (Albuquerque) is developing the BioXC 200GX system that integrates with the ICx MesoSystems AirSentinel trigger unit, and collects aerosol samples with a Cepheid GeneXpert PCR cartridge. Although the BioXC's sample-collection and autodispensing features minimize end-user handling, the PCR cartridge must be manually transferred into the GeneXpert analyzer for real-time amplification.

These technologies have improved detection times and achieved greater specificity and sensitivity of pathogen identification. However, they do not meet all of the Detect to Protect time-frame criteria of speed (less than 5 minutes), sensitivity, and specificity. The BioFlash biological aerosol collection and identification system by Innovative Biosensors (Rockville, MD), a next-generation bioaerosol monitoring system, has taken steps to achieve this goal.

## BioFlash® Detection System

BioFlash is a patented, self-contained system based on the Cellular Analysis and Notification of Antigen Risks and Yields (CANARY) platform, a diagnostic technology developed at the Massachusetts Institute of Technology (Cambridge, MA).<sup>13</sup> The core technology consists of engineered lymphocytes (B cells) that express membrane-bound, pathogen-specific antibodies and a cytosolic, calcium-sensitive bioluminescent molecule derived from the *Aequorea victoria* jellyfish (see Figure 2). Cross-linking of the antibodies by even minute amounts of a specific antigen (pathogen) leads to a rapid increase in intracellular calcium levels. In the presence of coelentrazine, the fully functional aequorin protein undergoes a structural change resulting in the emission of blue light that can be detected with a luminometer and without an external excitation source. The rapidity of the calcium response leads to the ultrasensitive detection of pathogens with PCR-like signal amplification in less than a minute (see Figure 3).

An extensive menu of B-cell lines has been developed for detecting pathogens in a variety of matrices, including clinical (e.g., urine, nasal swabs) and environmental samples

(see Table I).<sup>14–16</sup> While CANARY can be applied to a variety of samples, the BioFlash unit has been designed specifically for aerosol sampling.

<i>Bacillus anthracis</i> *	Spores, Vegetative
<i>Francisella tularensis</i> *	Vegetative
<i>Yersinia pestis</i> *	
<i>Vibrio cholerae</i> *	Strains O139 and O1
<i>Bruceella</i> spp.*	
<i>Borrelia</i> Toxin*	
<i>Bacillus subtilis</i>	Spores, used as simulant
Rift Valley Fever virus*	
<i>Listeria</i> *	
Rice*	
<i>Salmonella</i> spp.*	
<i>Shigella dysenteriae</i> *	
<i>Escherichia coli</i>	O157:H7
Foot and mouth virus*	
Venezuelan equine encephalitis virus*	
Dengue virus*	
Orthopoxviruses*	Smallpox
<i>Kalmanis</i> spp.*	
Potyvirus*	
<i>Phytophthora</i> spp.*	
<i>Chlamydia</i> spp.	
Methicillin-resistant <i>Staphylococcus aureus</i>	
Ovalbumin	

**Table I.** Available CANARY assays.  
\*CDC/USDA Category A or B List Agent

Table I. Available CANARY assays

The unit consists of a rugged, self-contained aerosol collector-and-detector into which BioDiscs are inserted (see Figure 4). Remote or manual triggering initiates aerosol collection into each of the BioDisc's 16 channels. Collection can be achieved in either static or turbulent environments through an internal blower that pulls 30 liters of air per minute (Lpm) through each of the 16 channels for a total sampling of 480 Lpm. The collected particles are dry-impacted into a groove in each channel, and the particle-depleted air is exhausted through a high-efficiency particulate air filter.

Each BioDisc is preloaded with preengineered B-cell mixtures, thereby eliminating fluid handling and reagent requirements, and minimizing end-user manipulation. Each BioDisc can be customized with respect to control samples and unique combinations of B cell mixtures.

The total multiplexing capacity per disc is more than 21 agents since 14 B cell storage chambers can be preloaded with up to three unique B cell lines that are capable of detecting three unique targets per channel, and redundancy can be built into each disc. In addition, two channels are reserved for positive and negative controls. This design provides for clear and rapid identification of pathogens detected within a given menu and minimizes the risk of false positives and false negatives.

One obvious concern with live-cell mixtures is maintaining the cells' functionality during the production phase, under a variety of storage conditions, and throughout the assay period. Once loaded into the BioFlash instrument, each BioDisc remains stable for up to one week at ambient temperature, which is suitable for integration into automated air-handling systems. While the exhausted BioDisc requires replacement after a triggering event initiates aerosol collection and the analysis is complete, the BioDisc is cost-effective and easy to replace. Refrigerating the BioDiscs provides greater long-term storage (about six weeks), and other advances have been made to increase the stability and shelf life of the B cells in the BioDisc.

Following aerosol particle collection, the BioDisc is spun, which dislodges a plug and releases the preloaded B cells from the storage chambers into the respective channels, bringing them into contact with the environmental samples. The BioDisc then slows down, and the resulting emission of light induced by the cross-linking between the collected particles (pathogens) and the antibodies expressed on the B cells is measured by a photomultiplier tube. The signal is interpreted using a detection algorithm and displayed on the face of the unit in an easy-to-read format, without any secondary hardware or software.

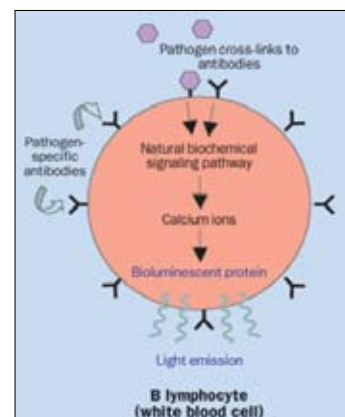


Figure 2. CANARY overview

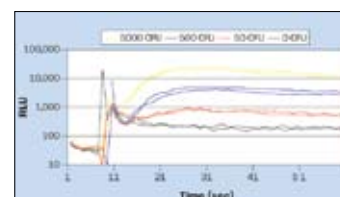


Figure 3. Sensitivity of CANARY assay using liquid biowarfare simulant agent.

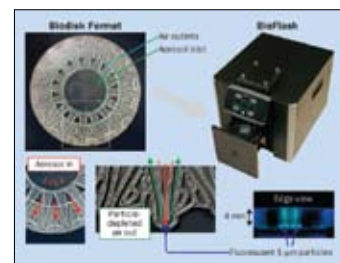


Figure 4. BioFlash and BioDisc overview

In field tests with a CANARY-based dry-collection bioaerosol assay, the technology was resistant to not only false alarms from interfering agents but also false negatives (see Table II). The technology detected about 200 colony-forming units per liter of air using *Bacillus subtilis* as the simulant (comparable to actual pathogens) in the presence of more than 40,000 particles per liter of contaminating particulates. Furthermore, the CANARY technology can detect as few as 20 agent-containing particles per liter of air (ACPLA) as demonstrated by using challenge samples during the course of 2012 on-site environmental challenges (see Figure 5). Receiver operating characteristic (ROC) curves illustrate a 98% probability of detection with a false-positive rate of less than 0.1% at 20 ACPLA (see Figure 5). These results illustrate the assay's sensitivity and specificity.

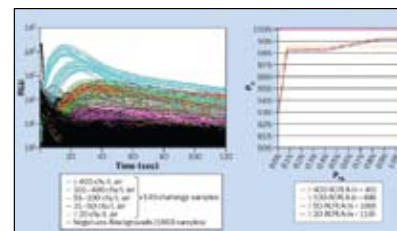


Figure 5. BioFlash test data

## Conclusion

The BioFlash biological aerosol collection and identification system is a comprehensive and affordable system. The system is also part of a small group of next-generation rapid pathogen detection systems that fully integrate aerosol collection and identification and have a compact footprint (about 1 sq ft), permitting practical deployment in building and site protection, military and civilian applications, port screening, and by first responders. BioFlash could be used in conjunction with a biological trigger, thereby providing continual monitoring via a tiered detection architecture, and rapid detection, confirmation, and identification of contaminating agents while minimizing the risk of false positives.

The advances made by all of the technologies discussed in this article have brought the notion of Detect to Protect much closer to practical realization. Within the next few years, more of these technologies will hopefully transition into readily available low-cost handheld devices, thereby further increasing their value.

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Interferent	Max particles per liter (ppL) tested	Elevated background? (Y/N)
Gravelly soil	100,000	N
Sandy soil	7,000	N
Dune sand	9,000	N
Silt	40,000	N
Montmorillonite	20,000	N
Loess	55,000	N
Peat moss	21,000	N
Muck	200,000	N
Arizona road dust	100,000	N
Dugway dirt	10,000	N
Diesel exhaust	84,000	N
Kaolin	100,000	N
Pollen (Pine)	10,000	N

**Table II.** CANARY assay resistance to interference.

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