Technologies to Enable Autonomous Detection for BioWatch: Ensuring Timely and Accurate Information for Public Health Officials : Workshop Summary

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Technologies to Enable Autonomous Detection *for BioWatch*

Ensuring Timely and Accurate Information for Public Health Officials

WORKSHOP SUMMARY

India Hook-Barnard, Sheena M. Posey Norris, and Joe Alper, *Rapporteurs*

Board on Health Sciences Policy

Board on Life Sciences

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Reviewers

This workshop summary has been reviewed in draft form by individuals chosen for their diverse perspectives and technical expertise, in accordance with procedures approved by the National Research Council's Report Review Committees. The purpose of this independent review is to provide candid and critical comments that will assist the institution in making its published workshop summary as sound as possible and to ensure that the workshop summary meets institutional standards for objectivity, evidence, and responsiveness to the study charge. The review comments and draft manuscript remain confidential to protect the integrity of the process. We wish to thank the following individuals for their review of this workshop summary:

Sara Beatrice, New York City Department of Health
Cindy Bruckner-Lea, Pacific Northwest National Laboratory
C. Rick Lyons, Colorado State University
Stephen S. Morse, Columbia University

Although the reviewers listed above have provided many constructive comments and suggestions, they did not see the final draft of the workshop summary before its release. The review of this workshop summary was overseen by **Enriqueta C. Bond,** Burroughs Wellcome Fund. Appointed by the Institute of Medicine, she was responsible for making certain that an independent examination of this workshop summary was carried out in accordance with institutional procedures and that all review comments were carefully considered. Responsibility for the final content of this workshop summary rests entirely with the rapporteurs and the institution.

Contents

Acronyms and Abbreviations

APDS	autonomous pathogen detection system
BAC	BioWatch Advisory Committee
BAR	BioWatch Actionable Result
BDS	biohazard detection system
CB	chemical biological
CDC	Centers for Disease Control and Prevention
Ct	cycle threshold
CTA	Chicago Transit Authority
DHS	Department of Homeland Security
DoD	Department of Defense
EPA	Environmental Protection Agency
FBI	Federal Bureau of Investigation
FDA	Food and Drug Administration
HHS	Department of Health and Human Services
HSMS	Hamilton Sundstrand CB mass spectrometer
IOM	Institute of Medicine
LANL	Los Alamos National Laboratory
LLNL	Lawrence Livermore National Laboratory

MALDI	matrix-assisted laser desorption/ionization
M-BAND	microfluidic bioagent autonomous networked detector
MS	mass spectroscopy
NG-ADS	next-generation automated detection system
NGS	next-generation sequencing
NHGRI	National Human Genome Research Institute
NORAD	North American Aerospace Defense Command
NRC	National Research Council
OHA	Office of Health Affairs (DHS)
PCR	polymerase chain reaction
PSU	portable sensor unit
REBS	resource effective bio-identification system
SPAMS	single-particle aerosol mass spectrometer
TRL	technology readiness level
USPS	U.S. Postal Service

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Introduction[1]

In 2001 the United States experienced the effects of bioterrorism firsthand when envelopes containing anthrax spores were sent through the U.S. Postal Service to several recipients, including two U.S. senators. In addition to the 5 deaths and 17 cases of anthrax infection that resulted, there were substantial economic costs and significant operational challenges in public health and health care, from the federal level down to the community level (Gursky et al., 2003). Partially in response to this incident, President George W. Bush in his 2003 State of the Union address announced that the federal government was "deploying the nation's first early warning network of sensors to detect biological attack" as one component of a new biodefense program (Shea and Lister, 2003). This network of early warning sensors is known as the BioWatch program.

> The BioWatch program, funded and overseen by the Department of Homeland Security (DHS), has three main elements—sampling, analysis, and response—each coordinated by different agencies. The Environmental Protection Agency (EPA) maintains the sampling component, the sensors that collect airborne particles. The Centers for Disease Control and Prevention (CDC) coordinates analysis and laboratory testing of the samples, though testing is actually carried out in state and local public health laboratories. Local jurisdictions are responsible for the public health response to positive findings. The Federal Bureau of

[1]The planning committee's role was limited to planning the workshop, and the workshop summary has been prepared by the workshop rapporteurs as a factual summary of what occurred at the workshop. Statements, recommendations, and opinions expressed are those of individual presenters and participants and are not necessarily endorsed or verified by the Institute of Medicine or the National Research Council, and they should not be construed as reflecting any group consensus.

> Investigation (FBI) is designated as the lead agency for the law enforcement response if a bioterrorism event is detected. (Shea and Lister, 2003)

In 2003 DHS deployed the first generation of BioWatch air samplers. The current version of this technology, referred to as Generation 2.0, requires daily manual collection and testing of air filters from each monitor. DHS has also considered newer automated technologies (Generation 2.5 and Generation 3.0), which have the potential to produce results more quickly, at a lower cost, and for a greater number of threat agents (IOM and NRC, 2009).

In response to a request from DHS, the Institute of Medicine (IOM) and the National Research Council (NRC) hosted a 2-day workshop that explored alternative cost-effective systems that would meet the requirements for a BioWatch Generation 3.0 autonomous detection system, or autonomous detector, for aerosolized agents (see Box 1-1 for the statement of task). This workshop, which took place June 25–26, 2013, in Washington, DC, was held under the aegis of the IOM Standing Committee on Health Threats Resilience, which assists the Office of Health Affairs (OHA) in DHS on a variety of issues related to its mission, including administration of the National Biosurveillance Center and the BioWatch program. Although the IOM and the NRC may be asked to examine other issues regarding BioWatch, the focus of this particular workshop was highly circumscribed, as was explained by William Raub, retired science advisor to the Secretary of the Department of Health and Human Services (HHS) and chair of the workshop planning committee, in his opening remarks. Raub further explained that the workshop was organized to examine the use of four classes of technologies—nucleic-acid signatures, protein signatures, genomic sequencing, and mass spectrometry—that could reach technology readiness level (TRL) 6-plus (in which the technology has been validated and is ready to be tested in a relevant environment [see Appendix E for TRL definitions]) over three different tiers of time frames: technologies that could be TRL 6-plus ready as part of an integrated system by 2016, those that are likely to be ready in the period 2016 to 2020, and those that are not likely to be ready until after 2020. "Our task," Raub said, "is to look at this exciting collection of technologies in various stages of emergence and distill them such that the DHS staff, as they work forward in planning for an autonomous detector, can make selections within the time frames and the many other constraints with which they are faced, not the least of which are budget and other considerations."

BOX 1-1
Statement of Task

The Institute of Medicine will convene an ad hoc committee to organize and plan a public workshop that will explore alternative cost-effective systems that would meet requirements for BioWatch as an automated detection system for aerosolized agents (alternatives to Generation 3). Systems identified need to be capable of being deployed by the Department of Homeland Security by 2018 and enable day-to-day environmental surveillance that would be of value to the public health and medical community. Specifically, the committee will develop the agenda for the workshop and identify and invite speakers and discussants to address the following questions:

- What are the advantages and disadvantages of alternative cost-effective, flexible, and accurate surveillance systems that could be deployed to ensure that an environmental biothreat is detected as soon as possible, generates a confirmatory response that is acceptable to the Centers for Disease Control and Prevention (CDC) and state/local public health officials, and thus optimizes the amount of time available for making the decision to deploy an available medical countermeasure?
    - Are there specific considerations for indoor vs. outdoor surveillance systems?
- What would need to be the specifications of a system that would be designed and deployed to enable detection of day-to-day functionality and value to the medical and public health communities to detect common threats (e.g., novel or known viruses, harmful volatile gases, etc.), as well as threat agents identified by through intelligence assessments?
- What is the current state of technology of biodetection systems using mass spectroscopy, and will the technology be advanced enough by 2018 that it could serve as an alternative to the currently planned polymerase chain reaction (PCR)–based system?
    - In addition to mass spectroscopy, are there other technologies that may be available to use in place of PCR to enable greater flexibility of the detection system?

The committee may commission white papers to help inform discussions at the workshop related to the aforementioned questions. The papers will be made available to workshop participants at the event or in advance. An individually authored summary of the presentations and discussions at the workshop will be prepared by a designated rapporteur in accordance with institutional guidelines.

WORKSHOP SCOPE AND OBJECTIVES

This workshop was designed to meet the following objectives:

- Develop an understanding of the nature of the biothreat and the role of biodetection.
- Discuss the history of the BioWatch program and the draft request for proposals released by DHS for alternative technologies for autonomous detection.
- Discuss the role of public health officials and laboratorians in the interpretation of BioWatch data and the information that is needed from a system for effective decision making.
- Review the current state of the art and explore the potential use of four families of technologies for the BioWatch program:
  - nucleic-acid signatures detected using polymerase chain reaction (PCR), microarrays, and other probe-based systems;
  - immunoassays and protein signatures;
  - genomic sequencing; and
  - mass spectrometry (MS).
- Explore how the technologies discussed might be strategically combined or deployed to optimize their contributions to an effective environmental detection capability.

The discussions, which were catalyzed by five white papers commissioned by the planning committee (see Appendixes F–J), also considered the deployment of potential technologies over three levels of readiness:

- Tier 1: fully autonomous biodetection systems, capable of 24/7/365 unattended outdoor and indoor operation, that will be at a TRL 6-plus by 2016.
- Tier 2: similar requirements but will not reach a TRL 6-plus level until sometime between 2016 and 2020.
- Tier 3: technologies that have the potential of meeting or exceeding the BioWatch requirements, but a fully autonomous, TRL 6-plus system would not be ready for deployment until beyond the 2020 time frame.

As Sally Phillips, acting principal deputy assistant secretary of OHA, said in her introductory comments, the goal of the workshop was to help her office “know what’s the right thing to do, what’s the possible thing to

do, and whether we can get there—today, tomorrow, and into the future." She asked the workshop participants to consider the question, "What should we be reaching for or what's just within our grasp that we need to carefully consider as we go forward?" She also reminded the workshop participants to remember during their discussions that BioWatch is not just about machines. "This is about states and locals having to make decisions about their communities, to save the lives of members of their communities in the best way possible."

ORGANIZATION OF THE SUMMARY

This publication summarizes the presentations and discussions that took place during the workshop, highlighting the key lessons presented, practical strategies for technology development, and the needs and opportunities for improving the BioWatch program. Chapter 2 provides an overview of the BioWatch program as a means of developing an understanding of the nature of the biothreat and the role of biodetection in countering that threat. Chapter 3 discusses the role and needs of public health officials and laboratorians in interpreting BioWatch results. Chapter 4 explores the state of the art and potential use of the four families of technologies being considered for the BioWatch program. Chapter 5 reviews the key themes and points made throughout the workshop and discusses how the four families of technologies might be combined or deployed strategically to optimize their contributions to an effective environmental detection capability.

2

Overview of the BioWatch Program

To begin the workshop, three speakers provided an overview of the BioWatch program and background on the nature of the bioterrorism threat. This chapter summarizes these presentations and the ensuing general discussion among the workshop participants. Several key points were highlighted in this session, including

- The biothreat has not diminished; it is evolving, becoming more "democratized."
- BioWatch, as part of a comprehensive approach, provides an opportunity to mitigate the consequences of a bioattack.
- Coordination and cooperation between federal, state, and local stakeholders is a requirement and a benefit of a successful BioWatch program.
- The aim of an autonomous detection system is to be "better, faster, and cheaper"; reducing time to response could save lives.

BIOTHREAT: A HISTORICAL OVERVIEW

Robert Kadlec, former special assistant to the President for homeland security and senior director for biological defense policy on the White House Homeland Security Council and now a consultant with RPK Consulting, discussed the historical background about bioweapons concerns. At the end of World War II, George W. Merck, who headed the nation's (now-defunct) offensive bioweapons program at the time, made a number of statements about the threat of bioweapons that still hold true today. Merck said then that it is possible to develop agents for biowarfare

without vast expenditures of money or the construction of huge facilities, that the development of bioweapons could proceed under the guise of legitimate medical research, that such weapons could overwhelm existing defenses, and that expenditures to counter the threat of biowarfare should be viewed in the light of the harm that might come to an unprepared America through a sneak attack. The program Merck headed was stymied, Kadlec explained, because the technology needed to ensure the safety of the researchers or to safely produce biowarfare agents on an industrial scale did not exist then.

Research continued, however, leading into the 1960s, the so-called golden era of U.S. bioweapons research; the program produced several agents and conducted both small- and large-scale environmental tests (e.g., validation tests for nonnuclear alternatives and atmospheric tests). These tests are germane to BioWatch, Kadlec said, because they demonstrated the feasibility of using environmental samplers to recover organisms released in large quantities into the environment. The United States terminated its bioweapons program in 1969 when President Nixon renounced the use of bioweapons and ordered the destruction of more than 71,000 dual-agent–filled munitions.

But although the United States ended its efforts, the Soviet Union continued its offensive biological warfare activities into the 1980s, Kadlec said. The Soviet program has been detailed in two books: *The Dead Hand*, for which author David Hoffman (2009) won the Pulitzer Prize in 2010, and, more recently, *The Soviet Biological Weapons Program: A History*, by Milton Leitenberg et al. (2012). During this period, the United States believed bioweapons would be used in low-intensity conflicts, such as the Vietnam War, with the intent of incapacitating people, not killing them. Experts also believed, Kadlec said, that biowarfare was a superpower-only capability.

In the 1990s, at the time of the first Gulf War, intelligence indicated that Saddam Hussein had a biological weapons program. Kadlec, who was a United Nations weapons inspector at the time, said that it was known that Iraq had started producing bioweapons in anticipation of its invasion of Kuwait in March 1990. “Iraq had probably 10,000 or more liters of liquid anthrax that they had distributed across the country in 1,100-liter spray tanks for aircraft,” he said. “How do we know that? We found them.” He showed a video clip from January 1990 of an Iraqi Mirage F1 jet spraying *Bacillus globigii*, an agent used to simulate biological warfare agents (Center for Research Information, 2004), in a final operational test to demonstrate the capability to release a large amount of

agent against a series of targets the Iraqi military had identified in Israel, Kuwait, and Saudi Arabia.

Based on computer simulations of the projected lay-down pattern, a single airplane fitted with one 1,100-liter tank filled with anthrax would have probably affected a significant number of American and coalition forces in Saudi Arabia. "Remember, there were 10 of these aircraft and the intent was to use all 10," Kadlec said. "It would have been a very significant event." The good news, he said, was that the U.S. Air Force claimed air superiority early in the conflict and prevented these planes from flying. The lesson to be learned from this experience was that Iraq was not a superpower, yet it had gained the capability to develop and use bioweapons.

Also during the 1990s, the Japanese religious cult and terrorist organization Aum Shinrikyo was producing biological agents and attempted, unsuccessfully, to conduct attacks with these weapons. What is significant about the near-miss, Kadlec said, is that the intelligence community and Japanese police authorities were totally unaware of this activity, just as the United States had been unaware of the size and significance of the Soviet Union's program.

Biowarfare took a new direction in 1998 when an al Qaeda's fatwa made specific mention of the use of strategic biological weapons. The organization created a laboratory dedicated to the development of bioweapons in Kandahar, Afghanistan, and it was in the process of attempting to make weaponized anthrax just prior to the attacks of September 11, 2001. This effort was overseen by Ayman al-Zawahiri, and there is no indication that al Qaeda has changed its strategic intent. Kadlec added that al Qaeda is known to have partnered with the Malaysian terrorist organization Jemaah Islamiyah, which has recruited a biotechnologist named Yazid Sufaat, who was trained at the University of Sacramento in the 1990s. The reality of the situation, Kadlec said, is that al Qaeda is more likely to procure a biological weapon than a nuclear weapon, and he added that other al Qaeda–affiliated groups have stated publicly that they want to use biological weapons.

As a final example to illustrate the threat of bioweapons, Kadlec discussed the anthrax attacks that occurred in 2001, which were not the work of al Qaeda but of one person, "a deranged scientist." In a sense, he said, this incident represents the final step in a trend stretching from the 1960s, when bioweapons were the province of superpowers, to today, when they have been "democratized." He noted that the terror organizations continue to promote the use of bioweapons and that the equipment

to produce pounds of anthrax can be bought over the counter for a few thousand dollars (Graham et al., 2008).

Returning to the 2001 letter-borne anthrax attack, Kadlec said that there were several cases of anthrax reported in the days before the first fatality, and he hypothesized that if today's environmental detection system deployed by the U.S. Postal Service had been in place then, it is likely that anthrax would have been detected before any of those cases had developed. "This doesn't say that environmental detection is the *sine qua non* silver bullet," Kadlec said in concluding his presentation, "but used in conjunction with better point-of-care diagnostics that can enable clinicians to make that diagnosis, I think we have a more comprehensive chance of dealing with and mitigating these events."

In response to a question about the effective human dose of anthrax, Kadlec said that one study suggested that 8,000 to 10,000 anthrax spores are needed to produce the disease in a human but that there is likely great variability in this number, depending on an individual's health and physiology (Cieslak and Eitzen, 1999). For other organisms, such as *Francisella tularensis*, which produces tularemia, an incapacitating dose is approximately 5 to 10 organisms. Intake of perhaps 10 times that many could result in death. Jeffery Runge then commented that it is important not to become fixated on anthrax because it is not the ideal agent for a bioweapon. "The requirement for an automated detection system ought to be able to turn on a dime and be able to measure agents that are deemed to be the highest risk," he said.

BIOWATCH PROGRAM HISTORY

Jeff Runge, former assistant secretary for health affairs and chief medical officer at the Department of Homeland Security (DHS) and now a principal partner at the Chertoff Group, said that his presentation on the history of BioWatch should serve as a context for considering how to develop the best possible system for protecting the nation, given the increased interest in biodefense compared with the situation when BioWatch was first developed. "History is, in fact, history," he said. "It's worthless unless it guides the future." He also commended DHS for its willingness to take a fresh look at the available technologies and not simply cling to the legacies of the past. Runge said it was his hope that the workshop would help DHS develop a requirements-driven balance in the face of what he characterized as a politically charged environment.

Runge stated that in the run-up to Operation Iraqi Freedom, an intelligence assessment found that there was an increased risk that Iraq would export biological weapons and that, without environmental detection, the United States would not be able to effectively initiate post-exposure prophylaxis to the population. Approximately 30 days after the program was conceptualized, BioWatch deployed its first-generation sensors, which were based on a commercial off-the-shelf product used by the Environmental Protection Agency (EPA) that was modified to detect biological agents using polymerase chain reaction (PCR) technology. These sensors were deployed in the highest-risk urban areas and required the federal government to create a distributed network for installation, maintenance, filter collection, and sample analysis. "As I look back at this," Runge said, "I think we can acknowledge that the requirement for speed afforded us less precision than optimal." At the time, he explained, the scientific understanding of how bioweapons might disperse over time in specific locations was not well developed, and a formal biothreat risk assessment had not been performed.

Because of the need for a speedy deployment, the Centers for Disease Control and Prevention (CDC) took an active role in providing guidance to local public health laboratories, which received supplemental funding from BioWatch to perform sample analysis. This effort was led by the White House Office of Homeland Security—BioWatch moved to the Science and Technology Directorate within DHS when DHS was created—and involved extensive interagency cooperation among the CDC, EPA, the Federal Bureau of Investigation, and numerous state and local government agencies, including public health, emergency management, law enforcement, and local environmental protection. "As you can imagine, this was a monumental task to do in a hurry and is actually an interesting feat in history," Runge said. At the same time, the Department of Defense (DoD), the U.S. Capitol Police, and the U.S. Secret Service established and maintained independent biosensor networks with little sharing of information about plans, systems, network design, technology, or results.

In April 2004 the White House issued Homeland Security Presidential Directive 10, *Biodefense for the 21st Century*, which was based on four pillars of activity: threat awareness, protection and prevention, surveillance and detection, and response and recovery (White House, 2004). Under this directive, DHS created the Office of the Chief Medical Officer and eventually moved the operations of BioWatch out of the Science and Technology Directorate and into the Office of Health Affairs

(OHA). The Science and Technology Directorate remains focused on research and development to support BioWatch autonomous detection.

Operational Challenges of BioWatch

From the program's beginning, Runge said, there were operational issues with regard to the decision making that would result from a positive BioWatch signal. The first issue involved jurisdiction. Terrorism is a national issue with national jurisdiction, Runge explained, but local governments are at the "tip of the spear—it's their skin in the game regardless of whose jurisdiction it is." As a result, early conference calls often had more than 100 people (representing local, state, and federal stakeholders) on the line with no uniform, consistent operational construct. Nonetheless, the system appeared to be working, and it led to a number of interesting discoveries. For example, BioWatch sensors revealed that there is *Brucella* in the air around county fairs and that atmospheric levels of *Francisella tularensis* increase during times of drought. Runge noted that if funding had permitted, immunological studies could have been conducted in these populations to further understand the nature of such patterns. However, rather than investing time and money to make these types of discoveries, he said, "our [DHS's] purpose was to protect the nation."

Speaking of the top-down approach that was taken when BioWatch was first established, Runge likened it to setting up North American Aerospace Defense Command (NORAD) radars around the country and asking local police departments to monitor the radars and call a national clearing office if they detected incoming missiles. Though the local laboratories welcomed the additional funding and added laboratory resources that initially came with BioWatch, the system was deployed with little local input, and the technology was not designed with the requirements of local laboratories in mind. As a result, he said, resistance to the program developed among many jurisdictions, and the program suffered from a lack of a consistently coordinated operational construct. There was also some loss of faith and support among local jurisdictions, although BioWatch did compel interactions among agencies and individuals who, historically, had never spoken to each other.

All shortcomings aside, the basic requirement for biodetection—the ability to maximize survival in affected populations—has not changed, Runge said. What has changed, though, is that the level of understanding about biological weaponry has become more sophisticated, and, as a re-

sult, the technology and overall system need to change to reflect the advancement of knowledge. One of the problems that technology must address is that the timeline of an attack has changed (see Figure 2-1), given advances in the ability to shape the size and characteristics of particles so that they penetrate deeply into the lungs. This technology, which Runge said is readily accessible over the Internet, has made the bioweapon threat greater. "High doses and small particle size truncate the incubation period to the point where the geographical distribution of casualties may look more like an incendiary or nuclear detonation, with a cluster around which we won't save anybody because of their dose," he said.

In response to the current understanding of the characteristics of bioweapons, DHS has issued a set of requirements for BioWatch Generation 3 that includes autonomous detectors with technology that is at least as sensitive and specific as the current system using PCR. The detectors

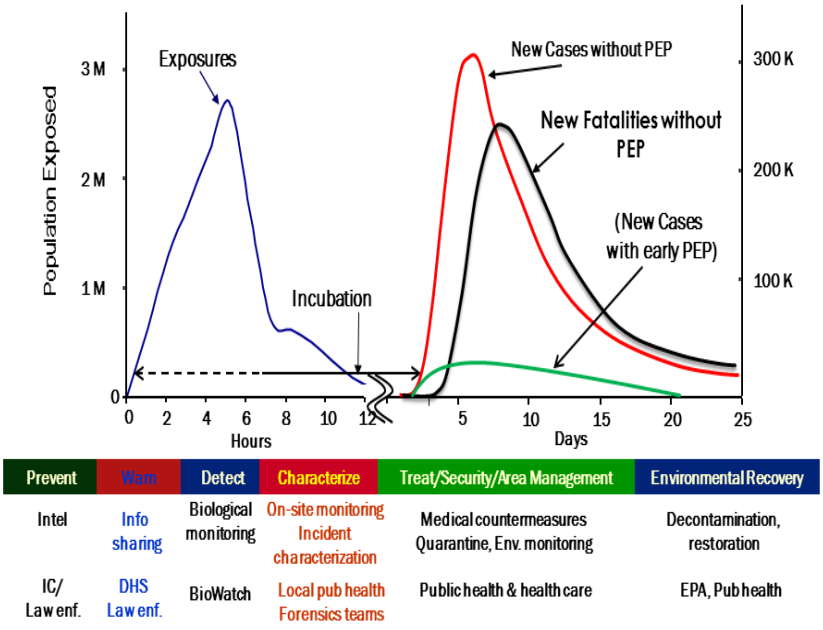

FIGURE 2-1 Normalized timeline of an aerosolized anthrax attack.
NOTE: DHS = Department of Homeland Security; EPA = Environmental Protection Agency; IC = Incident Command; PEP = postexposure prophylaxis.
SOURCE: Runge presentation, June 25, 2013.

must operate in continuous mode to reduce response time and should be able to preserve the captured live agent to enable fingerprinting and forensics, Runge said. The cost targets at the time the requirements were issued were $50,000 per unit and $10,000 per unit in annual operational and maintenance costs. Runge said that it is important to note that the responders to the Generation 3 request for proposals were mainly smaller technology companies and defense and aerospace companies; the broader biomedical industry was not well represented. He also mentioned that a planning scenario for New York City put the cost of an aerosol anthrax attack at $1 trillion.

Runge briefly discussed the development of what was called Generation 2.5, a multiplexed assay that was designed to serve as an interim solution that could be deployed indoors on a limited basis. A few machines were purchased and deployed in New York City, again with limited coordination between federal and local agencies. An inconclusive result from an autonomous unit produced a crisis of confidence within the New York police department counterterrorism unit, and the system was turned off. At the same time, CDC declared at a national BioWatch meeting that it had no confidence in this multiplexed assay. As a result, no other machines were deployed.

In closing, Runge said that several important points can be drawn from the history of BioWatch. Above all, the timely detection of biological agents is crucial as the first step in enabling the timely deployment of medical countermeasures to the public, agent identification, and immediate notification and simultaneous situational awareness for federal, state, and local officials. Detection delays greater than 24 hours severely limit the usefulness of any biodetection system, as does a distributed network with scores of owners. Runge said that in his opinion, “We need central authority and central control, but with full integration and buy-in from local operational authorities.” From an operational perspective, all signals from any detector will require some adjudication by human experts, which suggests that sensors of various methodologies, costs, and discrimination may be useful in a layered warning system. Finally, he said, two important lessons from history are that careful joint operational planning provides ownership of the endeavor and that the hardware to accomplish the mission should be tailored to planning and training.

CURRENT BIOWATCH PROGRAM AND AUTONOMOUS DETECTION

Michael Walter, detection branch chief and BioWatch program manager in OHA at DHS, began his presentation by reminding the workshop audience that the BioWatch mission came out of President George W. Bush's 2003 State of the Union address and was established by executive order. Its mission is to detect a biological attack, identify the agent used, and prepare a response in time to minimize the impact of the attack. Walter described it as "a very straightforward mission, but very complex to integrate and accomplish" and added that, over time, a number of other tasks have been tacked onto the program. Initially, BioWatch was deployed in 10 high-value cities, but program officials were soon asked if the system could be used for other high-value targets, such as events that might be under threat. They were also asked to accomplish the program's mission better, faster, and more cheaply, which, Walter noted, was the impetus for the workshop. BioWatch is a state-of-the-art system, but it is labor-intensive and therefore expensive, so there are drivers to move from a manual to an autonomous system.

One area that has received a great deal of focus during the past few years has been the effort to provide guidance at the federal, state, and local levels about how to respond to BioWatch information. "We all know that there is no way to respond to a bioterrorism event on the fly," Walter said. "This has to be planned out. Everybody has to have an understanding of what you are doing and what your actions are going to do to affect other agencies that are going to be responding." Furthermore, BioWatch does not exist in a vacuum, so it must be interoperable with other systems that have been deployed by other agencies, Walter reiterated.

Currently, BioWatch is deployed in more than 30 jurisdictions nationwide, and it employs more than 150 people working in the field, more than 130 in laboratories, and more than 100 public health professionals who are integrated with the program, in addition to the dedicated program staff that organizes and coordinates the program. For the most part, BioWatch sensors are deployed outdoors with limited indoor operations. BioWatch sensors are deployed on a case-by-case basis at special events affecting national security (e.g., a presidential inauguration) and local special events such as parades, marathons, and even the Super Bowl at the request of local jurisdictions. Walter noted that although there has been controversy about what constitutes a false-positive, the program maintains that from a technical, analytical, and operational

standpoint, there has never been a false-positive. "We have been right 100 percent of the time," said Walter. "What we have seen on our filters is there, and we have confirmed that with the CDC."

Reiterating what Runge said earlier, Walter said that BioWatch depends on partnerships, and he added that he considers the state and local BioWatch Advisory Committees (BACs) the crowning glory of the program. These committees, he explained, force organizations that do not generally talk to each other, that often do not like each other, and that compete for funding with one another, to sit around the table and figure out how to deal with specific situations. These BACs have become the nexus for all hazard response committees and serve as a bridge not only between the federal government and local authorities but also between state and local agencies. He said that the BACs and BioWatch program officials have established a coherent, organized, and disciplined notification process that "lets us know what happened, where it happened, what's being done about it, and how we are responding to it, plus what is needed from the federal government to start the response." None of this structure will change when BioWatch transitions to an autonomous detection system, he said.

The key to the program, Walter said, is the planning and preparedness (see Figure 2-2) that will enable local jurisdictions to be the first to respond to a bioterrorism attack, with local authorities applying their expertise and knowledge of their own cities and counties. BioWatch assists in planning and preparedness by providing guidance documents that point out various considerations that the local authorities can use as they formulate their plans. Walter noted that the current structure for planning and preparedness activities was designed to address the chaotic operational structure that existed in the program's early days.

BioWatch also provides training and test exercises so that local jurisdictions can see if their plans work. "If we do those two things correctly—if we plan and prepare and we train and exercise based on whatever technology is available—then deploying technology becomes more transparent and more effective for the state and local governments that have to use it," Walter said. He added that public health agencies tend to be conservative about new technologies, so these activities are important for building trust and allowing public health officials to become comfortable with new technologies when they are deployed. Both planning and preparedness activities and training and exercise activities are coordinated by the public health and preparedness group in the BioWatch program office. Included in this office are jurisdictional coordinators who support

the BACs and help shoulder the organizing activities that state and local public health authorities would otherwise have to manage.

Through these activities, BioWatch program officials have developed a good idea of how individual jurisdictions are going to respond to an attack, but there is concern about how the system will operate in the face of multiple attacks or when an autonomous system that collects as many as 10 samples per day from every machine is deployed, Walter noted. "We need to get in front of that now, because we're not going to catch up once we start a deployment," he said.

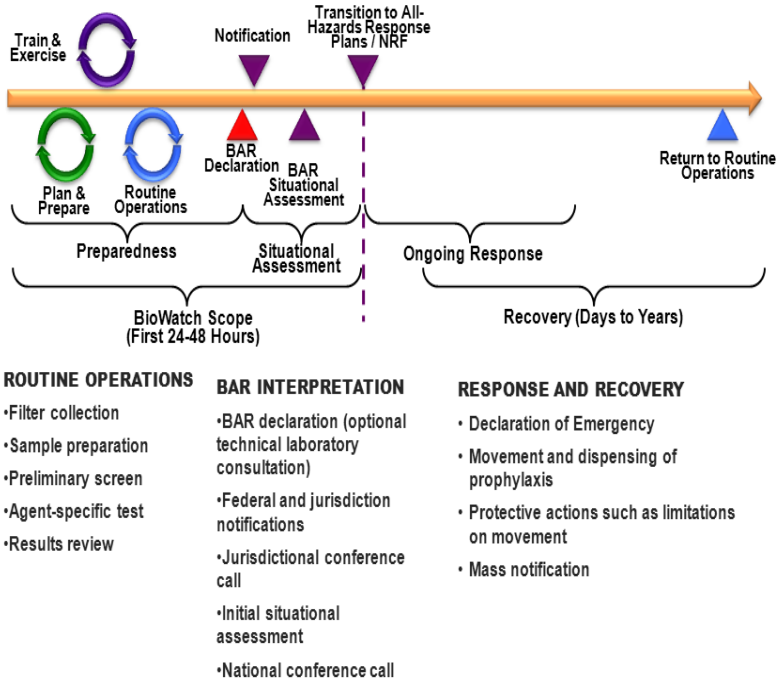

FIGURE 2-2 BioWatch program scope.
NOTE: BAR = BioWatch Actionable Result; NRF = National Response Framework.
SOURCE: Walter presentation, June 25, 2013.

Operational Components of BioWatch

The daily operations of the program have three components: field operations, laboratory analysis, and public health and preparedness. At its root, Walter said, BioWatch is a tool of the public health domain. Field operations center on what is essentially a vacuum cleaner pulling air through a filter. This system is dependable, inexpensive, and easy to deploy, but it requires a person to go out every day to collect the filters and bring them to the laboratory according to strict standard operating procedures. Adherence to these standards is checked by independent audits by an outside evaluator.

The laboratory operations are the most sophisticated part of the BioWatch program. All analyses are performed using PCR that identifies target organisms through DNA signatures. The assays themselves are designed by DoD's Critical Reagents Program and CDC's Laboratory Response Network. Walter characterized these assays as being thoroughly validated, very specific, extremely sensitive, and highly dependable. Based on the results from those assays, the local laboratory director or designee can declare a BioWatch Actionable Result (BAR). Declaring a BAR is not a federal event, he noted, although CDC and the BioWatch program office can help with a technical discussion as to whether the assay results make sense. "But in the end, it is the state and local laboratory director that declares the BAR and initiates the response," Walter said. The analytical strategy is straightforward, he added. A primary screen serves as a low-pass survey for organism identification. Positive results are followed by a verification panel that provides expanded analysis with highly discriminating DNA signatures.

Walter explained that a BAR does not necessarily mean that a terrorist attack has occurred or that a viable biological agent has been released (see Figure 2-3). It also does not mean that an agent is infectious or that there is a risk to the public's health. What a BAR does mean is that a filter collected from a specific location contains genetic material from an organism that is tested by the BioWatch system. This result is actionable only after assessment by the state and local BioWatch advisory committee.

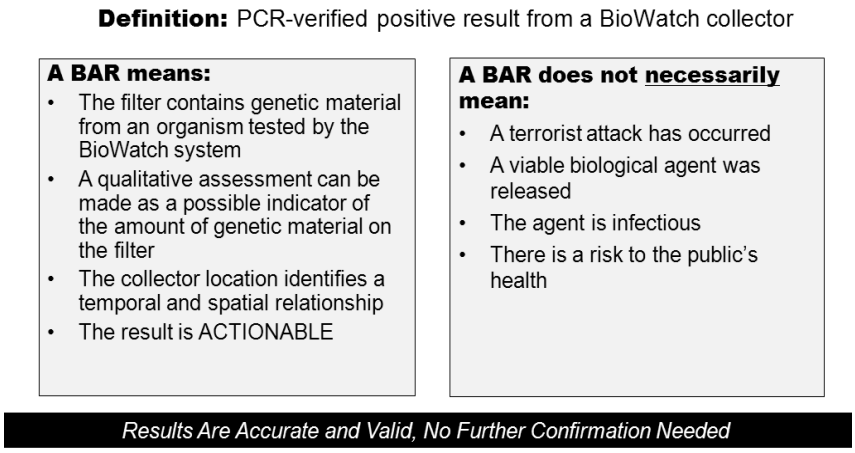

FIGURE 2-3 A BioWatch Actionable Result.
NOTE: BAR = BioWatch Actionable Result; PCR = polymerase chain reaction.
SOURCE: Walter presentation, June 25, 2013.

Future Autonomous Detection Systems

In the future, BioWatch plans to develop an autonomous detection system, which, Walter noted, is not necessarily the same as the Generation 3 program that has been put on hold pending an independent analysis of alternatives, which is currently ongoing. The primary reason to move to autonomous detection is time—the faster that an attack is detected, the more lives can be saved (see Figure 2-4). An additional reason is that autonomous detection would increase system capabilities and coverage without sharply increasing costs. Current technology, he said, can cut the casualty rate almost in half compared with a situation in which there is no detection system in place. Autonomous detection could reduce the casualty rate even further by cutting the time between when exposure occurs and when a BAR is declared. With the current system, the time between release and declaration of a BAR is somewhere between 12 and 36 hours. Newer technologies developed in the first phase of acquisition can produce results in 3 to 6 hours (see Figure 2-5). Walter noted that this reduction in the time it takes to get results "could dramatically decrease the time between potential exposure and confirmation of the result, giving more time back to state and local public health . . . for a more effective response."

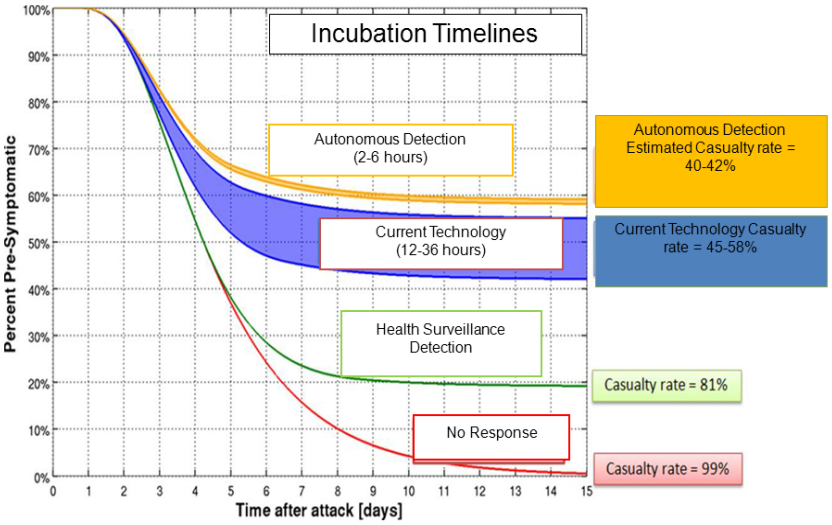

FIGURE 2-4 The primary benefit of early detection through autonomous detection lies in reducing the casualty rate and number of fatalities following a release.
SOURCE: Walter presentation, June 25, 2013.

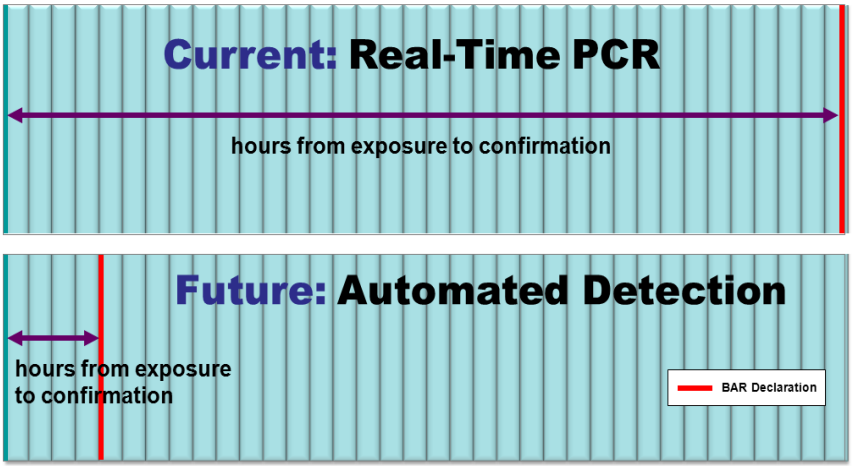

FIGURE 2-5 Comparison of detection timelines: Event-to-detection and confirmation.
NOTE: BAR = BioWatch Actionable Result; PCR = polymerase chain reaction.
SOURCE: Walter presentation, June 25, 2013.

One effect of successfully developing an autonomous detection system will be that operational concepts will need to be redone in coordination with state and local public health authorities to account for the increased sampling and broader geographic coverage, noted Walter. "We need to make sure response plans are effective, based on that technology," Walter said in his closing remarks. "We need to make sure we have exercises that link the technology and the response. The only way we will have a successful deployment of any technology is going to be if state and local public health have confidence in that system, which means we have to have a robust quality assurance program backing it up, and we need to be able to prove at the state and local level that these machines see what they are supposed to see and they don't see what they are not supposed to see."

DISCUSSION

Commenting on Walter's remarks, John Vitko, rector of St. Luke Church and former director of Biological and Chemical Countermeasures for the DHS Science and Technology Directorate, reiterated that the deployment of an autonomous system will not change the fact that local public health officials will still be responsible for declaring a BAR. He also stressed that CDC and DHS officials will still be available for consultations prior to declaring a BAR.

A workshop participant asked how BioWatch shares costs with local jurisdictions. Walter said that BioWatch has contractors who work for the program in local laboratories but the program does not cover laboratory costs at the state and local level. BioWatch does provide funding to collect filters and deliver them to the laboratories as well as to cover the costs of planning training exercises. Eric Eisenstadt, an independent technical consultant, asked about the uniformity of responses to a BAR across local jurisdictions. Walter responded that there are slight differences reflecting local situations but that the initial response across jurisdictions follows a template and occurs on the same timeline. Eisenstadt also questioned use of the term "BioWatch Actionable Result" as potentially confusing and wondered if there might be a need to change the language. Walter responded that all state and local authorities involved in the BioWatch program are familiar with this term and have a shared, clear understanding of what it does and does not mean. He noted, too,

that his office consulted with NORAD regarding the term "BAR" and was told that it was satisfactory.

Raymond Mariella, Jr., a senior scientist at Lawrence Livermore National Laboratory, asked how long it would take to develop a new assay for an autonomous system for a previously unidentified organism. Walter responded that it could take a year or longer because, in addition to the assay itself, there would need to be accompanying bioinformatics. Although reagents can be ordered quickly through CDC and DoD, the primary reagent suppliers for the BioWatch program, Walter said that validation is needed to ensure that the assay is working accurately, and this process could take a substantial amount of time.

3

Public Health's Perspective on the Role of BioWatch in the Decision-Making Process

Local and state public health agencies sit on the front lines of the BioWatch system. Any autonomous detection system will have to meet the needs of those agencies in order to instill confidence in the results from such a system and to enable public health officials to make the appropriate decisions following declaration of a BioWatch Actionable Result (BAR). To provide the workshop with a public health perspective, Sandra Smole, director of the Division of Molecular Diagnostics and Virology in the Bureau of Laboratory Sciences at the Massachusetts Department of Public Health, summarized the key points she made in the commissioned paper that she had written as background for this session's discussion (see Appendix F). Five panelists then discussed the role of public health officials and laboratorians in the interpretation of BioWatch results.

Session moderator Suzet McKinney, deputy commissioner of the Bureau of Public Health Preparedness and Emergency Response in the Chicago Department of Public Health, said that the panelists had been asked to consider two questions when they prepared their remarks:

1. What factors related to BioWatch autonomous detection will affect decision-making response actions after a BAR is reported?
2. How will jurisdictions determine if action steps are indicated in response to a BAR, and what should those action steps be?

This chapter summarizes the presentations and the ensuing general discussion among the workshop participants. Key points include

- The technical data that lead to a BAR are just one part of the information that is needed; context is important for decision making.

- Public health officials are in the position of making high-regret decisions in the face of uncertainty—the information provided must be accurate and reliable.
- Public health stakeholders—as the end users—want to be engaged at all stages (design, development, and testing).
- Desired features and considerations for a detection system include
  - faster detection time;
  - remote access;
  - instrument-specific performance data;
  - information on agent identity, viability (alive or dead), and susceptibility to medical countermeasures;
  - spatial resolution (information about release location and amount); and
  - archived sample data for later testing.

INFORMATION NEEDED FOR DECISION MAKING

In her overview of BioWatch as it relates to public health, Sandra Smole stressed that BioWatch is viewed in the public health community as a surveillance tool that is used in conjunction with other tools, such as syndromic surveillance, for detecting the presence of an infectious agent or toxin of public health significance. In that respect, information from BioWatch contributes to and is interpreted within the context of the larger surveillance picture. As was mentioned in an earlier presentation, no two BioWatch jurisdictional operations within the 30-plus locations where BioWatch is permanently deployed are exactly alike.

As a laboratorian, Smole said, she sees the definition of a BAR as having two components (see Figure 3-1). In the laboratory setting, a BAR is the production of a reliable, reportable laboratory result, backed by a laboratory-quality system. In the operational setting, a BAR is a determination that a result is actionable within the context of other key pieces of information. She said that autonomous detection technology has the potential to improve the depth of information provided by the BAR by, for example, improving pathogen identification and strain discrimination capabilities. It also has the potential to provide a quantitative BAR result that offers useful information for those considering the response to multiple collector "hits" and developing an appropriate response, particularly with respect to indoor and transportation venues.

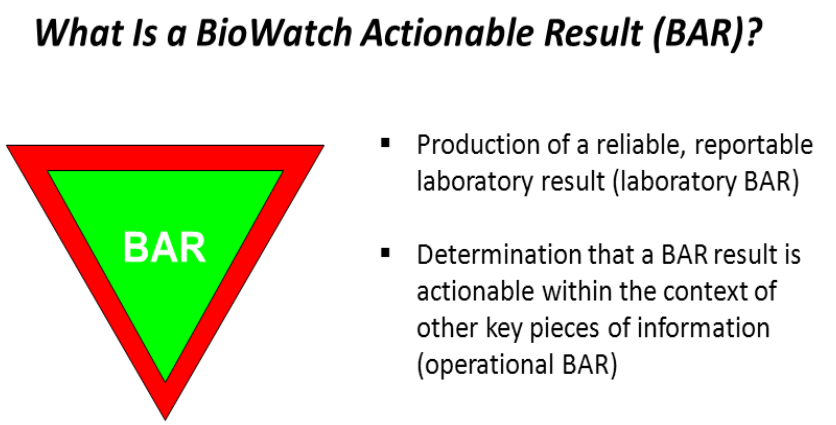

FIGURE 3-1 Two components of a BAR: laboratory and operational.
SOURCE: Smole presentation, June 25, 2013.

Smole said that from a public health perspective, the biggest issue stemming from the use of autonomous detection systems will be the need to manage a new timeline for response actions that will result from an increase in testing frequency. Any new technology will have to come with a robust quality-assurance program and a secure data-management infrastructure that will be capable of handling input from each instrument within a more extensive network, Smole noted. She continued by saying that an autonomous detection system will need to provide flexibility in the panel of agents that it can detect; to allow for varied collection duration or shorter time between samplings, particularly after a BAR; and to be able to store a portion of a test sample for additional verification by other test methods. It will be important for local jurisdictions to be able to perform basic maintenance of the detectors.

Concerning the information needed to determine a BAR from a new autonomous detector, Smole said that the public health laboratory director or designee will need prior knowledge of parameters such as sensitivity, specificity, reproducibility, robustness, and inherent limitations that will be critical for interpreting test results. Once an instrument provides a positive signal, the public health laboratory director or designee will need access to instrument performance indicators, positive and negative controls, threshold settings, and historical data from the detectors. She noted that while BAR data may indicate a detection event, they will still require interpretation within the context of other information.

Smole also said that any new technology should be benchmarked against the current technology. It is important to remember, she said, that data from the current system have served to establish a baseline for sensitivity, specificity, reproducibility, and an understanding of the inherent limitations of the current technology as far as interpreting and acting upon results. "There is a specific DHS [Department of Homeland Security] protocol in place on our BioWatch portal that describes the mechanism for introducing a new platform and/or method to the BioWatch Program. It should be assumed that the new technology must perform equal to or better than the current technology before it is adopted." As a final comment, Smole added that, in her experience, public health is supportive and poised for adoption and implementation of new technologies to protect the nation's health.

FOUR REGIONAL PERSPECTIVES

Houston, Texas, and Surrounding Harris County

Two of the strengths that public health agencies bring to the table are their versatility and their ability to make decisions even when sufficient information is not available, said David Persse, emergency medical system physician director in the City of Houston's Public Health Authority, who serves as the BioWatch Advisory Committee (BAC) chair from Houston. It is important, he said, that the technology community understand the nature of the job that he and his counterparts in public health take on regarding BioWatch, particularly the time pressure they will be under to make critical decisions from the moment they receive a notification from the laboratory that there is a BAR. From the time Persse is notified of a BAR, he has an hour to gather further intelligence; put the BAR in context, given that intelligence; and arrange a phone call with the appropriate local officials, including the local office of the Federal Bureau of Investigation (FBI) and the Joint Terrorism Task Force, his colleague Umair Shah from Harris County, and the jurisdictional coordinator from the state of Texas, who will also need to have gathered information by the time of the call. During that call Persse will solicit input from his colleagues, a key benefit of the BioWatch program, which encourages collaboration between different agencies, but the final decision as to whether the BAR signal indicates a threat to public health rests with him. "This is not a group decision or a democracy," he said, "there is a

health authority that is responsible for the lives of the people within that jurisdiction."

One hour after the local call, Persse and the other appropriate officials in the jurisdiction prepare for the national call. "At the end of that phone call, we're either going to do something or not do something [e.g., obtain additional samples, temporarily close facilities, alert local hospitals, begin prophylaxis, etc.], but at that point, from our perspective, BioWatch ends, and the response begins."

Today, Persse and his colleagues in public health have a degree of comfort with the current Generation 1 and 2 system because of their familiarity with the way that the cycle threshold (Ct) values from the PCR assays are reported and with the peculiarities of each of the portable sampling units (PSUs) located in their community. A Ct value indicates that a certain DNA sequence (representing a target organism) is present at a given location—and a higher Ct value corresponds to lower concentrations of the agent detected—but how it got there and what it means must be interpreted in the context of other information. In Houston, for example, some of the portable sensor units are more active than others because of an organism that is endemic to the area, and some are affected by wind and weather patterns or even the season or time of day. All of this information helps Persse put a BAR in context (see Box 3-1).

In a perfect system, Persse said, BioWatch would identify the organism, determine whether it was alive or weaponized, and provide forensic information that would pinpoint who released it—something that he acknowledged was not realistic. "The BioWatch system is much like a clinical laboratory test," Persse said. A clinical lab test provides a set of numbers that have to be interpreted in the context of the individual patient, regardless of how sophisticated the test is or which technology was used to run the test, and the same is inevitably going to be true for BioWatch, no matter how sophisticated it becomes, he noted. "As much as I would like for an autonomous detection system to be able to tell me that we have a terrorist attack with this organism, this much of it distributed at this time, in this location, that's not going to happen," Persse said. "I have to take the information that's given to me, put it into context, and move forward."

BOX 3-1
Variables Analyzed to Put a BAR in Context

- BAR—yes or no?
- Agent detected
- Number of collectors positive
- Day, month, year
- High, low, and average temperature
- Heating degrees (65 − Avg = # of heating degrees). Use when the average temperature (Avg) is less than 65.
- Cooling degrees (Avg − 65 = # of cooling degrees). Use when Avg is greater than 65.
- Departure from normal temperature, degrees Fahrenheit
- Precipitation for the day, inches
- Precipitation total up to and including the day for 2, 3, 4, 5, 6, and 7 days prior
- Snowfall depth (?)
- Fraction of daytime minutes with sunshine (1 to 10 scale)
  - 0 = overcast conditions all day
  - 5 = partly cloudy
  - 10 = sunny all day
- Average and maximum wind speeds
- Average/resultant wind direction
- Gust wind direction
- Degrees of change in wind direction
- Weather codes (thunderstorm, mist, haze, fog, etc.)

NOTE: BAR = BioWatch Actionable Result.
SOURCE: Persse presentation, June 25, 2013.

Art of Decision Making

Umair A. Shah, executive director of the Local Health Authority, Harris County (Texas) Public Health and Environmental Services, stated that although the workshop was focused on the science of biodetection, he wanted to address the art of decision making. "This is not just a science, but it's what we as clinicians and public health practitioners do all the time, which is really taking the contexts, the nuances, and making that part of our decision-making process," he explained. "Public health decision making is still an art based on the experiences of the individuals and the agencies that are part of the process and done in the contextual framework of the available information."

In Harris County, which is the third most populous county in the nation and one that encompasses a diverse set of communities in an area the

size of Rhode Island, making a decision after being notified by Houston public health of a BAR requires considering those detection data, along with information from disease surveillance and contextual intelligence. Disease surveillance includes examining zoonotic patterns reported by local veterinary clinics and the state zoonosis surveillance system as well as data on human disease patterns that may have been reported by area hospitals or other health departments. Contextual information includes details about environmental patterns and unusual security threats or security patterns.

While the decision-making process is occurring, the county begins mobilizing its crisis risk communication resources and makes sure that its operational support functions are ready. County officials also take fiscal constraints into account when making a decision on how to respond to a BAR. Fiscal constraints, Shah said, are having a real impact on the value proposition today. For example, a decreased investment in the technologies that enable syndromic surveillance and automated disease reporting, combined with decreased staffing for surveillance, is diminishing response capabilities, which in turn makes the decision on how to respond to a BAR even more art than science.

Shah concluded his remarks by offering some considerations for those in the audience who are involved in developing and deciding on Generation 3 or other future BioWatch technologies. The first thing to think about, he said, is that a laboratory positive is not the same as a public health positive, and the issue of false-positives is likely to be a bigger issue with a new autonomous detection system with more cycles, more tests, and more results on a daily basis. Second, a biodetector that has the capability to automatically signal a BAR without human input or additional context (red light/green light) may be appealing from a technology perspective, but from the public health perspective such a feature would take away the ability to engage in nuanced decision making.

It is also important, he said, to remember that the integrity of public health is critically important. "How does the public view us if we do launch or don't launch a response based on incorrect or incomplete information?" Shah said. He added that he shares Persse's view that the BioWatch BAR system is like a clinical laboratory test.

> As a clinician, if I had a woman who walked through the door and I said, "You have a spot on your mammogram, and I'm going to immediately send you for a bilateral modified radical mastectomy, based on that spot," immediately, my days as a physician are numbered. That is the challenge here. What we are really trying to do is take that spot on a

> mammogram, figure out what other diagnostic and contextual information we need to put to the puzzle, and then figure out what to do with that information.

Persse said he strongly agreed with that view, adding that the only currency public health has is credibility. The bottom line, Shah said, is that any new technology must make public health more effective.

The San Francisco Bay Area

The San Francisco Bay area covers more than 8,000 square miles and is inhabited by a highly mobile population of 7.2 million people living in both urban and rural communities, said Erica Pan, deputy health officer and director in the Division of Communicable Disease Control & Prevention, Alameda County (California) Public Health Department, adding that San Francisco itself is one of the smallest counties in the region with a residential population of less than 800,000 that doubles during the day. In total, the health jurisdictions of six counties, including San Francisco, and the city of Berkeley are included in the San Francisco Bay Area BAC. One unusual feature of the Bay Area is that the regional BioWatch laboratory is the California state laboratory, which is not in the Bay Area region. Furthermore, while San Francisco does not have any indoor detectors, San Francisco International Airport does, and a representative of the airport is also a member of the advisory committee.

The BAC has developed a decision matrix to discuss during the local conference call and guide its response to a BAR (see Table 3-1). The matrix, developed from local experience, ranks the likelihood of a potential problem organism in the environment after a BAR. For example, a BAR resulting from the detection of *Francisella tularensis* with a Ct value greater than 35 by a single detector is rated as an unlikely release event because *F. tularensis* is an indigenous organism that has been detected previously in this BioWatch region. By contrast, a BAR resulting from a single detector identifying an organism not previously seen by BioWatch in the region would be rated as a probable release event, as would a BAR resulting from the detection of *F. tularensis* with a Ct value less than or equal to 35. A BAR would also be judged to be a probable release event if additional collectors provided positive signals or if high concentrations of the organisms were detected. At that point, Pan explained, the advisory committee looks for other information, such as the

TABLE 3-1 San Francisco BioWatch Advisory Committee Decision Matrix

Likelihood of Organisms in the Environment	Example of BioWatch Actionable Result (BAR)
Unlikely	Single collector positive of organism not previously detected by BioWatch in the region, or *F. Tularensis* (FT) with a Ct value >35
Possible	Single collector positive of organism not previously detected by BioWatch in the region, or FT with Ct value ≤35
Probable	Single collector positive of organism not previously detected by BioWatch in the region and the following information *may* increase the risk of release: a. Additional collectors with confirmed positive signals b. High concentrations of agent detected c. Positive environmental surface swipe sample(s) d. Recent outbreak of human or animal cases in the region e. Overt unusual/adversarial activity consistent with threat f. Evidence of weaponized or genetically modified agent g. Recent bioterriorism incident or credible threat in the United States h. FBI-designated special event near positive collector

SOURCE: Pan presentation, June 25, 2013.
NOTE: Ct = cycle threshold; FBI = Federal Bureau of Investigation.

presence of positive environmental surface swipe samples, recent outbreaks of human or animal disease in the region, overt unusual or adversarial activity consistent with a threat, evidence of a weaponized or genetically modified agent, a recent bioterrorism incident or credible threat in the United States, or an FBI-designated special event near the positive collector.

Decision making is aided by a detailed list of organism-specific criteria that account for factors such as incubation period, transmissibility between humans, past incidence of human disease, the likelihood of natural environmental occurrence in the region, and the potential risk to public health. At that point, the BAC orders immediate environmental testing, using a plan developed by its Environmental Protection Agency (EPA) and FBI partners, and it may declare a potential local emergency while waiting for input from other state and federal agencies. Pan said that the BAC has discussed increasing the frequency of retrieving filters from detectors, but it decided that such an action would not be useful

with current technology and given the limited laboratory capacity for testing.

Other decisions that will be made on a case-by-case basis include how to conduct public health surveillance, either with enhanced passive techniques that involve sending out a health alert to emergency medical staff and clinicians to be on the alert for symptoms of a certain disease or via active surveillance with teams visiting hospitals to search for cases. Although veterinary surveillance would also be useful, Pan said that the region has few resources in this area; this is one area in which the BAC would like to expand the available resources.

The advisory committee will also need to consider post-exposure prophylaxis for the filter retrieval staff and local emergency responders, Pan said, and it will have to alert the personnel that will be needed to coordinate a potentially large medical surge. Another important decision will be whether to issue an alert to the Strategic National Stockpile and to regional staff that would be needed to distribute medical countermeasures. Pan said that the Bay Area BAC has had internal discussions as well as talks with other advisory committees around the country about what kind of exposure reduction recommendations it would make. She noted that her committee has decided that the distribution of post-exposure prophylaxis will take priority, and it has worked closely with the Bay Area Mass Prophylaxis Working Group to create a unified screening algorithm for all of the region's points of distribution and also an associated website that would go live immediately after a release event was confirmed.

Thoughts on Future Technology

Regarding the impact of newer technology, Pan reiterated earlier remarks that it will be essential to build confidence in the results that is at least equal to the comfort level that public health has developed with the current technology. "We would like to see equal sensitivity and specificity and some ability to have some oversight or understanding of the quality control of any autonomous detection," she said. More frequent testing periods would provide more timely notification and potentially reduce the window of likely exposure and the number of people exposed, which in turn would reduce the demand for prophylaxis. This would be particularly true in the case of indoor detectors, where faster detection would decrease how far someone who was exposed would have traveled. One

potential downside of more frequent testing may be an increased possibility of false-positive test results.

Pan concluded her remarks by listing other information that new technology might be able to provide that would help public health's ability to respond to a BAR. Her wish list included

- quantitative results;
- the ability to distinguish between pathogenic species, closely related non-pathogenic species, and environmental species;
- rapid confirmation of viability;
- antibiotic susceptibility;
- indications of weaponization or intentional genetic modification;
- feasible decontamination strategies; and
- the flexibility to include or remove organisms from the screening panel.

Pan noted that public health has been calling for studies to identify endemic organisms that make up the environmental background. "We have found some of them incidentally through this program," she said, "but it would be much more useful if we knew for sure the background data for certain endemic organisms in different areas." She also reiterated Shah's earlier remarks about the impact of budget constraints on the public health infrastructure and said that because of the dramatic cuts to public health funding, it is difficult to find qualified people to fill positions that are available. Developing an anthrax vaccine does no good, she said, if there is no one available to distribute it.

New York City

In his presentation, Colin Stimmler, director of the BioWatch Program, New York Department of Health and Mental Hygiene, said that it is important to remember that the goal of BioWatch is not just to detect an airborne biological agent for the sake of detection. "It is detection to respond, to hopefully save lives," he said. In New York City, only 40 percent of the BioWatch portable detection units are outdoors, with the remaining 60 percent in indoor locations. Another unusual feature of the New York City BioWatch network is that it is administered by the New York City Police Department, with the Department of Health and Mental Hygiene serving as the lead scientific agency responsible for testing, environmental sampling strategies, disease surveillance, and implementing

the public health response plan. All told, there are 16 agencies in the New York City Stakeholder Group, that area's version of a BAC.

Indoor Detection

New York City has had indoor detectors since 2003, and their location is based on police department threat assessment, not on the percentage of the population covered. A sizable percentage of the indoor detectors are in transit hubs that are managed by seven stakeholders, Stimmler said, with some of those stakeholders reporting to regional authorities and not New York City. Regardless, he said, all of those stakeholders must be integrated into planning and also into any response. Stimmler also noted that filters are collected multiple times per day from the indoor transit hub detectors. To put the importance of these indoor collectors in context, he noted that the busiest airport in the world—the Atlanta international airport—processes about 250,000 people per day, while three times that number pass through New York City's Grand Central Station daily, and more than 5 million people use the New York City subway system every workday.

With that information as background, Stimmler presented a scenario as a means of illustrating the challenges that New York City public health faces because of its extensive indoor program. In this scenario, which he characterized as the one that is most likely to present him and his colleagues with the necessity of making an immediate, potentially high-regret decision, the public health laboratory reports a BAR for a biological threat agent from an indoor detector in a major transit hub. At that point, a number of things are known: the number of detectors reporting a BAR, the location of the detector or detectors reporting a BAR, the Ct values, the sample period range for each detector, the performance of the detectors in terms of sensitivity and specificity, and syndromic surveillance data from the previous day. What will not be known is whether there has actually been a release, if the organism is viable or not viable, and, if it is not viable, whether it is dead because it was dead upon release or because it died from desiccation on the filter. Other unknowns will include

- whether the release is an isolated incident or part of a series of incidents;
- when or where the release occurred;
- how and how much of the material was released;

- where the material has migrated since release;
- whether the material was altered or weaponized and
  - whether it is more or less virulent,
  - has a longer or shorter incubation period,
  - is resistant or susceptible to medical countermeasures, or
  - if the size of the particles makes them more conducive to transport; and
- whether there has been an error in the analysis of the sample despite all of the safeguards to prevent such a mistake from happening.

With those gaps in mind, there are many possible explanations for a single detector reporting a BAR. One possibility is that there was a large, point-source release that then dispersed and became a series of line-source releases. This situation has the potential for wide dissemination, and a possible explanation for only a single detector having reported a BAR is that this one detector is identifying the edge of the large-scale release. Another, far more benign possibility is that a vendor selling products made from animal hides set up his or her display right in front of the portable detector. These two possibilities have two vastly different consequences, yet there will be no way of knowing if either is true when public health has to report a BAR. In fact, Stimmler and his colleagues assume that the only information available at the time of reporting a BAR will be the laboratory results.

The stakeholder group's initial response in this scenario will be to seek answers to four questions: Has there been a release of a biological threat agent? If there was a release, where, when, and how did the release occur? Who might be at risk of infection? How can the stakeholder group target the available public health interventions to protect the greatest number of potentially at-risk people? Answering the first question, Stimmler said, leads to the default hypothesis upon which all other actions will be based, but given the limited information available at that moment, he said, "all of our actions are potentially high-regret and are instantly public."

The unique challenge of having an indoor program is that there will have to be a decision about closing the facility in which the signaling detector is located, Stimmler said. To help with that decision, environmental sampling teams in protective gear will make an additional collection of samples with surface wipes and a retrieval of the detector filters—a very public display that is unavoidable. Stimmler then made what he characterized as the understatement of the year: "If we get this wrong

and we overreact, the economic and political impacts are huge. If we get it wrong and underreact, the public health impacts of doing nothing are even bigger."

With that scenario and its consequences in mind, Stimmler discussed how BioWatch Generation 3 or autonomous detection would change the situation. Currently, the time between potential release and detection by New York City BioWatch ranges from 9.6 to 37 hours, which means the system is basically one that detects in order to treat. Autonomous detection could reduce that time to 2 to 4 hours, which would turn the system into one that detects to protect. An autonomous system could make it possible to respond quickly enough to limit additional exposures and make it easier to estimate who was affected by the release. However, any new technology needs to have as close to zero percent false-positive and false-negative rate as is possible. Because of the scale of the New York City BioWatch network, even a 0.01 percent failure rate—which Stimmler said would translate to two false-positive BARs per year—would be unacceptable, given the consequences.

Moving into the realm of what he acknowledged might be science fiction, Stimmler provided a public health wish list for Generation 3 or autonomous detection technology that was very similar to the list Pan presented. Additional items on his list included the ability to resolve the time (in minutes) and the location of the release as well as estimates of the aerosol mass that was released.

Chicago

The Chicago Transit Authority (CTA), the second largest transit system in the nation, moves 1.6 million people per day among 145 rail stations and 11,500 bus stops. The CTA has its own BioWatch response plan because, as John Plante, senior manager of emergency preparedness for the CTA, described the situation, the system's 1,200 railcars are not only a target but could also serve as a vector for transmitting a released bioweapon. The system connects the city; contamination could spread not only within the subway, but to surrounding streets, businesses, and the airport (see Figure 3-2). This indoor response plan is based on a number of assumptions, including that every one of those railcars will have to be inspected, that detection in the subway system might result in cessation of operations for a period of time, and that detection in the sub-

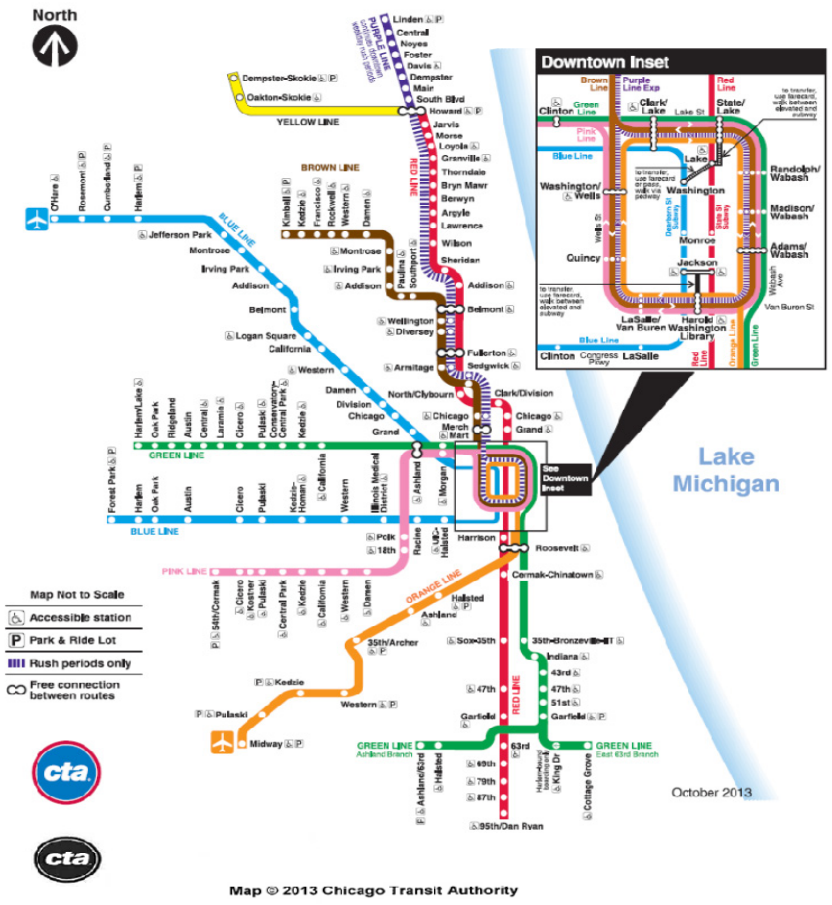

FIGURE 3-2 The CTA rail map.
SOURCE: Chicago Transit Authority.

way system may necessitate decontaminating not only the railcars, but also the platforms, ancillary rooms, and other structures.

Once a decision is made that affects the subway system, the response will include the management of patrons, management of employees, management of the transportation system, management of the facilities involved, and risk communication. Plante said that the city has practiced an evacuation scenario and has consulted with both the Department of Defense and the EPA on how to test and decontaminate a railcar. He said

that the mayor holds the final authority to declare an event but that the CTA, with advice from the city's department of public health, maintains control of the subways throughout the closure and evacuation process. The CTA, he added, will follow the advice of the Chicago Department of Public Health regarding whether or not to keep the rail system running.

Plante reiterated what the previous panelists had said regarding the need for an autonomous detection to be as accurate and reliable as the current system. The key for any new technology, he said in closing, will be to provide fast, accurate information that will enable the end users—managers such as himself—to make a decision about what is happening, how bad it is, what to tell customers and employees, if a facility will need evacuation, and how and when the affected facilities can be reopened.

DISCUSSION

To open the discussion, Eric Gard, a scientist with the Defense Biology Division at Lawrence Livermore National Laboratory (LLNL), asked the panelists to talk about when they would use atmospheric models of potential exposure regions and backward models to establish the point of release. He also asked them if current information limits the utility of those models in either the outdoor or the indoor environment. Persse replied that these models are used early on, almost as soon as the initial notification of a BAR is received. He said that his team has a mechanism in place to feed data to the team's federal partners to start the modeling process and added that modeling might be more of a benefit to law enforcement than to public health because in the event of a release, everyone who wants prophylaxis is going to receive it. Pan agreed with those comments but thought that modeling may help if it comes to prioritizing who gets vaccine in the case of supply limitations.

Raymond Mariella, Jr., from LLNL noted that all of the panelists mentioned the desire to determine if an organism was alive or dead when detected and asked if there is a particular time frame that would be useful for making that determination. Smole said that it is possible now to produce that information in 24 to 48 hours, but her impression was that the information would be more useful if it was available in a matter of a few hours. Stimmler said it would be most helpful if the time frame was minutes rather than hours because that determination affects the decision to release medical countermeasures and trigger procedures to prevent the spread of an infectious agent.

In response to a question by Thomas Slezak from LLNL about networking an autonomous system, Smole said that it would be important for the laboratory to be part of any such data communications network, and Persse said that any network would have to be transparent. "If there was a delay between when the feds found out and when the locals found out, you are going to create an air of mistrust," he said. Plante added that it was essential that any network be robust and secure.

Robert Kadlec asked about the importance of including the ability to detect antibiotic resistance in future BioWatch developments, and Pan replied that antibiotic resistance is a basic and important piece of information that decision makers need when they make a request to the Strategic National Stockpile. Shah agreed with that comment and added that such information allows public health to make a more nuanced decision in a timely manner.

Session rapporteur Beth Maldin Morgenthau, assistant commissioner for the Bureau of Policy, Community Resilience and Response within the Office of Emergency Preparedness and Response at the New York City Department of Health and Mental Hygiene, voiced her concern that a system that produces results every few hours could strain what are already limited and diminishing public health resources and said that the BioWatch program needs to keep that in mind as it contemplates rolling out the next version of this technology. She also raised the point that although everyone agrees with the need to have flexible panels whose biological agents can be added or removed as needed, it will be necessary to have a response plan in place before adding a new agent to the panel. Again, with diminishing resources, it is important to consider how public health will respond to new agents. Maldin noted, too, that as BioWatch is deployed in additional indoor settings, it will be important to remember that the public will be more aware of the system when public health moves into a setting to gather additional information needed to declare a BAR. She then reiterated Plante's points about the difficulty in cleaning up after a release and the difficulty of determining when to declare a contaminated area clean.

Maldin also said that she agreed with Smole's and Persse's comments about the need for transparency with any networked system. "Our labs should look at the results first, then public health should get to talk about the results before anyone else sees them," she said. Shah noted that ultimately the decision of how to respond to the release of a biological weapon must be a local one. "Our citizenry, our residents, expect local governance and local decision making," he said, which implies both a

need for transparency and a need for local public health offices to manage the data from a networked system.

Donald Prosnitz, an independent consultant, asked the panelists if they would take BioWatch into their jurisdictions today, given what they know now and the experiences they have had with BioWatch. Plante said that he would because, for all its limitations, BioWatch is still better than syndromic surveillance in terms of shortening the time for detection and saving lives. Stimmler said yes as well but added that he would want to have more input in terms of the agents being tested for and the logic behind those choices. Pan agreed, but said she would insist on more local involvement at the beginning of the rollout process. She added that resource constraints today would make BioWatch seem like an unfunded mandate, a sentiment that McKinney seconded. Persse agreed that BioWatch was valuable and said that he would have it in his jurisdiction again because, in addition to the information it supplies, it has also created a network among state, local, and federal officials that is valuable, a comment that Smole seconded. Shah was the most reluctant, saying that at the time BioWatch was originally deployed, there was no good understanding of what a BAR meant. However, assuming that state and local officials have more input and information as future programs are deployed, he said he is looking forward to the autonomous detection system. He noted that people need to view it in the context of all the tools available in the surveillance toolkit and also recognize that resources are limited.

4

Potential Technologies for the BioWatch Program

Having been provided with an overview of the BioWatch program and input from public health officials that run local BioWatch programs and use its information for critical, potentially high-regret[1] decision making, the workshop participants discussed the current state of the art, exploring the potential uses of four families of technology in the BioWatch program, and considering how these technologies might be strategically combined or deployed to optimize their contributions to an effective environmental detection capability. For each of these four sessions, the writer of a commissioned paper presented an overview of the technology being discussed (see Appendixes G–J). Each presentation was followed by three or four talks on potential next-generation technologies and then an open discussion among the workshop participants.

Each of the panels in the four sessions focused on autonomous, fully automated, end-to-end systems that would be technology readiness level (TRL) 6-plus ready over three time frames: by 2016, by 2020, and beyond 2020. Panel members were also asked to identify the major integration issues in making these systems field-deployable and capable of detecting organisms of interest with the required sensitivity and specificity to meet the needs expressed earlier by public health officials. This chapter summarizes those presentations and the ensuing open discussions among the workshop participants. Key points during the four sessions include

[1]In this context, "high-regret" refers to a situation in which a decision maker would encounter large negative consequences for being wrong.

- Potential challenges to consider:
  - Integration of multiple components or technologies ("detection is done").
  - Effectively maximizing sample concentration and minimizing sample contamination (sample to environmental background).
  - High-quality databases, bioinformatics tools, and assay validation are needed for all technologies.
- A multidimensional approach is encouraged:
  - Technologies using immunoassays and mass spectrometry are fast; nucleic-acid approaches and genome sequencing can provide specificity and identify novel threats.
  - Orthogonal testing increases the reliability of a BioWatch Actionable Result (BAR) and confidence in the system.
- Extensive and repeated field testing will be necessary for a detection system.
- It is important to involve the end user (public health) in system development and testing.
- The Department of Homeland Security (DHS) was encouraged to look beyond the familiar technologies and development pathways.

AUTONOMOUS DETECTION SYSTEMS USING NUCLEIC-ACID SIGNATURES

In the first of the four sessions, Raymond Mariella, Jr., a senior scientist at Lawrence Livermore National Laboratory (LLNL), provided an overview of his commissioned paper reviewing the state-of-the-art technologies available for detecting organisms using nucleic acid signatures (see Appendix G). The following topics were then discussed by the panelists: lessons that could be learned from other industries in autonomous detection and how those lessons might apply to BioWatch; the evolution of assays to include and exclude various threats; the current state-of-the-art system used by the U.S. Postal Service (USPS) and next-generation autonomous detection system based on multiplex polymerase chain reaction (PCR) assays; and systems concepts and integration issues.

Nucleic-Acid Signatures at Three Levels of Readiness

In his interpretation of the three tiers of readiness, Mariella said that any system that would be deployable at TRL 6-plus by 2016 would have

to consist of off-the-shelf components and be running today. For Tier 2—those ready by 2020—it should be possible to improve sample preparation and add another level of identification to the automated assays, perhaps through some sort of targeted amplification of virulence regions. For Tier 3 systems, those available after 2020, technology developers still have years of vigorous research ahead to create and validate assays, develop the necessary informatics software and data interfaces, and perform extensive integration work.

Mariella then provided his notional overview of the system components and their states of readiness (see Figure 4-1). For Tier 1 readiness, the technologies that are available today and are at TRL 8 or TRL 9 (see Appendix E for TRL descriptions) include those for high-volume aerosol collection into an aqueous medium, sample collection, and primary detection. For collection, virtual impact collectors can reduce sample contamination and determine the particle size distribution of the particles that it collects. It should also be a simple matter to add a video camera to the system to monitor external events that might explain a positive signal. Wetted-wall and other high-volume particle collectors can increase sample yield and deposit particles directly into aqueous media that can be divided into aliquots for multiplexed analyses. Collecting samples directly into a liquid would also increase the odds that any microorganisms would remain alive for subsequent viability testing. Automated systems that include PCR for nucleic acid analysis, which require the lysis of spores to release nucleic acids, are currently the only technologies ready for 2016 deployment. For primary detection, Tier 1–ready technologies can divide samples into aliquots for single-target, real-time PCR or can conduct multiplexed assays using bead-based capture probes, Mariella said.

Tier 2 systems could incorporate a light-scattering unit that would log the ambient particle count, size distribution, background fluorescence, and other information that provides context for any positive signal. Enrichment chemistries and amplification methods for nucleic acid target sequences could improve sample preparation in ways that allow for virulence assays that would increase the signal-to-noise ratio of the system. It may also be possible by 2020 to add 400-base sequencing of targeted sequences to an autonomous system and to incorporate resident expert systems for preliminary data analysis and two-way communications with monitoring. One potential technology that Mariella mentioned that could be available after 2020 would use a surface molecular recognition reagent or a pool of reagents to capture organisms of interest to enrich samples for subsequent analysis (Hou et al., 2013).

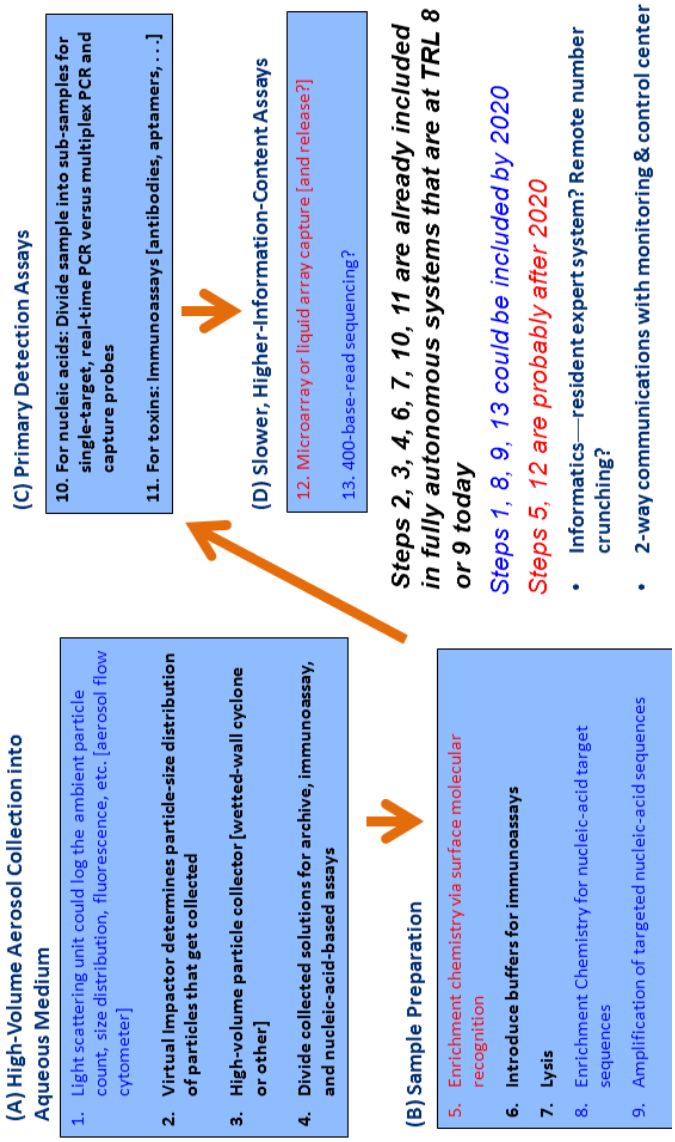

FIGURE 4-1 Notional: General system overview.
NOTE: TRL = technology readiness level.
SOURCE: Mariella presentation, June 25, 2013.

Today, Mariella said, two systems are ready for deployment: the Autonomous Pathogen Detection System (APDS) developed largely by LLNL (Regan et al., 2008) and the Microfluidic Bioagent Autonomous Networked Detector (M-BAND) developed by Microfluidic Systems Inc., a subsidiary of PositiveID (Sanchez et al., 2011). Mariella said that both systems have been tested successfully in the field. "If you want to put something in the field this afternoon, you have those two to choose from," he said. He characterized a suite of aerosol-characterizing equipment as being mature and at TLR-8 or higher, including an inexpensive simple aerodynamic particle sizer and more expensive systems that use fluorescence measurements to characterize particles. The most advanced system that is ready for deployment uses an aerosol flow cytometer to capture particles and feed them into a mass spectrometer for further characterization.

Mariella quickly described the use of selective reagents for capturing pathogenic bacteria and viruses using antibodies, aptamers, synthetic peptides, or nanolipoproteins. In his opinion, he said, it should be feasible to use a panel of 20 or 30 reagents on magnetic beads to capture all of the threat organisms, separating them from the background atmospheric microbial content and thereby improving the performance of the downstream analytical technologies. He also mentioned the AmpliSeq technology (Murphy et al., 2005; Towler et al., 2008), which can be used to search for targeted amplification of virulence regions or other identifiers, including plasmids in bacteria, which again would provide better signal-to-noise performance. Emulsion or digital PCR could also improve both single and multiplexed amplification and provide intrinsic quantification of starting copy number, offer real-time detection in fewer cycles with less amplification bias, and be available by 2020 (Kiss et al., 2008; Shiroguchi et al., 2012; Vogelstein and Kinzler, 1999; Whale et al., 2013; Woyke et al., 2010).

Cindy Bruckner-Lea from the Pacific Northwest National Laboratory commented that one driver of technology that had not been mentioned was the need to analyze indoor samples more efficiently. She suggested that particle detection could be used to trigger the more costly sample analysis. Mariella questioned this suggestion, saying that, in his view, it would not provide the best return on investment.

Issues and Opportunities for BioWatch Autonomous Detection

According to Allen Northrup, sample processing and fluidics are the two areas related to next-generation BioWatch systems that need the most attention. "In my opinion, detection is done," he said. "The point is to get the sample into the detector, and there are plenty of state-of-the-art, FDA [Food and Drug Administration]-approved detection technologies for doing that." Fluidic control has its challenges—mainly related to situations in which the output of one module may not match the input needs of another module—but industries other than biodefense, particularly the food and beverage and pharmaceutical industries, have solved this problem and have created proven commercial systems using fluidics.

The key challenges for any system, he said, are to define specificity within a defined limit of detection and to then define sensitivity within the context of that specificity (see Figure 4-2). False-positives and false-negatives, he said, also need to be defined within a predetermined limit of detection, with the caveat that, at the limits, false-positives and false-negatives are inevitable with all analytical systems. "The question becomes what can we live with? What is realistic?"

PCR is a well-proven, reliable technology that can provide quantitative results in a multiplexed system. The challenge for the next-generation BioWatch system, Northrup said, is to automate it and improve handling of the complex samples that are collected in the field. "However, the thing I learned today is that BioWatch is more than instruments and threshold cycles," he said. "It is a basis of human interaction and communication, informed decisions, and ancillary information. Whatever we build and deploy is going to have to have that human interaction." In his opinion, a lab-in-a-box system that inputs a sample and outputs a result will not be well accepted by public health, which will want to see the direct result of any analysis and understand thoroughly the analytical parameters. Northup recommended that system developers provide public health agencies and laboratories with the subcomponents of their systems for evaluation and approval early in the development process.

To conclude his remarks, Northrup said that the BioWatch community needs to test new systems repeatedly and be tolerant of early failures. He proposed that BioWatch should be developed as a modular system with significant, ongoing input from public health laboratories concerning which processes they would like to see automated. The resulting automated modules should then be provided to the laboratorians for

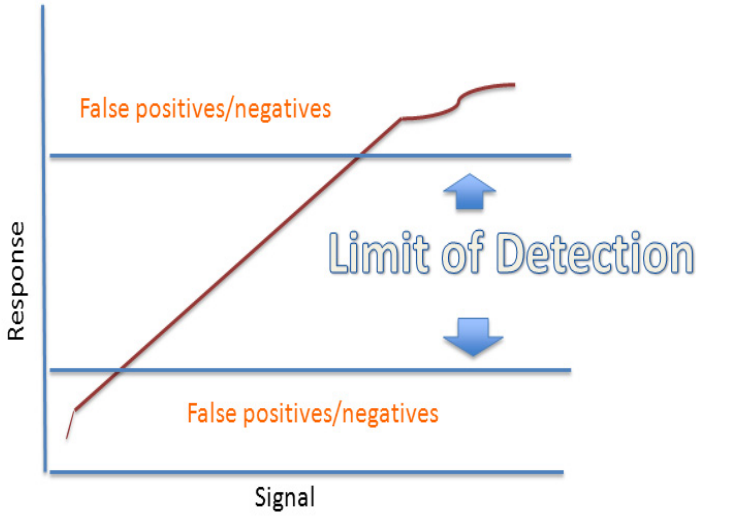

FIGURE 4-2 Fundamentals of all instrument analyses.
SOURCE: Northrup presentation, June 25, 2013; Northrup Consulting Group.

validation alongside currently accepted methods in an iterative process that yields a robust, proven module for incorporation into an automated system for which public health will have already developed familiarity and confidence.

Assay Development and Evolution

In the next presentation, Ivor Knight, senior vice president and chief technology officer of Canon U.S. Life Sciences, Inc., focused his remarks on the question: How will the technology evolve signatures as increasing knowledge of targets and neighbor strains reveals both false-positive and false-negative situations? As background, he described how such signatures are developed today, starting with identifying genes that are distinctive to a target organism and developing probes or primers to detect those genes. This deductive approach, which has been used success-

fully for more than 25 years, relies on deep knowledge of the pathogen in question and works well for targeting key pathogenic determinants, such as toxin-coding genes. However, this approach ignores most of the genome as a potential signature candidate.

The newer genomic approach, which has been developed in the past decade or so in parallel with the revolutionary advances in genome sequencing technology, starts with a target organism's full-genome sequence data and compares that sequence with the full-genome sequence data from all other organisms. The result is a set of sequences that are unique to the target. This approach requires no knowledge of the pathogen, and the analysis is done completely *in silico*, yielding multiple targets. Knight noted that this approach relies on coherence between the *in silico* world of genome databases and the real world, where false-positives and false-negatives arise. As the number of microbial sequences continues to increase, he said, signature designs will continue to improve, lowering the probability of false-positive and false-negative results.

During the past decade, this type of approach has also yielded a new understanding of bacterial and viral genetics. One thing that has become apparent, Knight said, is that there is not a clear-cut situation in which a sequence is found in the target and not in the background genomes. "What we have learned is that microbial genomes form a continuous genetic background," he said. Instead of each species having a completely unique genome, species share what Knight called pan-genomes, core genomes that are common to all members of a particular genus of bacteria, with various additions and subtractions of genes and gene families across the species and strains of species in that genus (Hu et al., 2011) (see Figure 4-3).

As a result of this new understanding of microbial genomes, it is now necessary to consider pan-genomes and to use multiplexed capabilities to interrogate more potential genomic signatures. "We need multiple targets to be able to assess differences on this continuous genome space," Knight said. Moreover, target sets will have to be adaptive, and analytical systems will have to accommodate changing signatures on what Knight suggested could be a quarterly basis. "We're going to want to change the signatures so that we can have greater accuracy in our detections," he said. For the 2020 scenario, Knight said, it will be necessary to sequence not just signature regions but the entire genetic complement of a sample in order to provide a probability-based analysis of the genomic content of the sample.

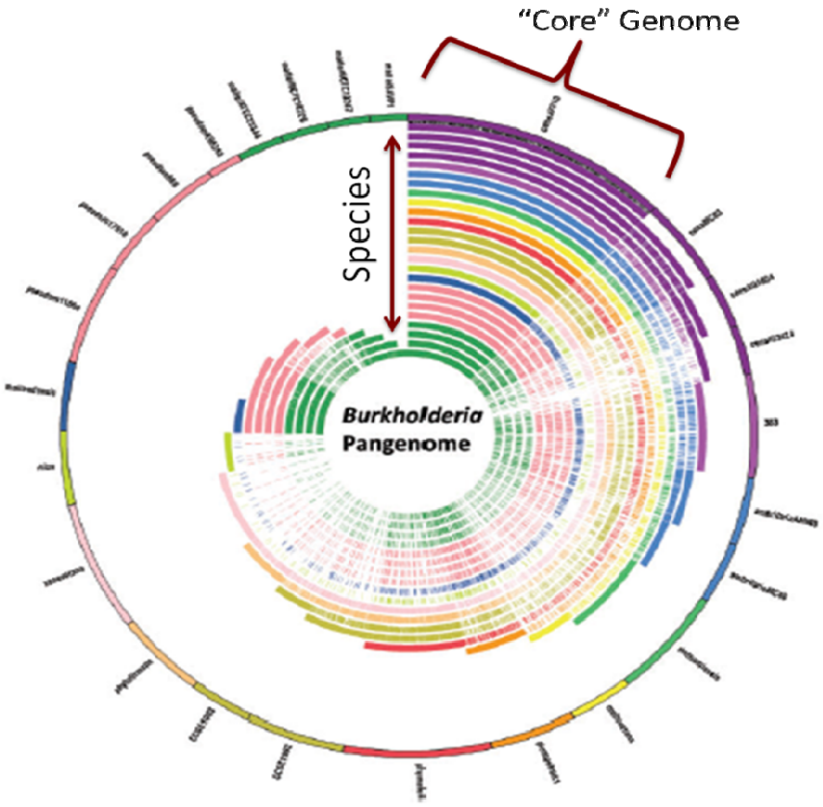

FIGURE 4-3 Pan-genomes of the genus *Burkholderia*.
SOURCE: Knight presentation, June 25, 2013 (adapted from Hu et al., 2011).

In his opinion, Knight said, PCR-based multiplex technologies using bead arrays and real-time PCR with or without hybridization probes are mature and will be ready to be deployed by 2016, while digital PCR will be ready for deployment by 2020. The big challenges ahead, he said, will involve system integration and workflow. For example, how will reflexive testing, in which an initial screen for a single target provides a potential positive result that triggers additional, deeper testing, be integrated into an autonomous system? Knight also noted that consumables handling has not yet reached the required level of maturity for it to be ready for widespread deployment in an autonomous system in a way that would enable laboratorians responding to a positive result to instantly trace the history of the consumables in an instrument all the way back to

their manufacturers. Similarly, systems monitoring and preventive maintenance procedures will need to be developed. Finally, reiterating Northrup's earlier recommendation, Knight said that all of these systems and procedures must from the beginning be worked out with the laboratorians involved. "If public health laboratorians are going to be involved in decision making, they are going to want to know and understand the quality system that was put in place for that automated system. They are going to want to see that the quality system is the same or similar or analogous to the quality system that they are used to in their laboratories that has been developed and is tried and true over the years."

State-of-the-Art and Next-Generation Autonomous Detection Systems

Next, in a talk on Northrop Grumman's work in the biodefense field, David Tilles, vice president for CBRNE [chemical, biological, radiological, nuclear, and explosive] Defense at Northrop Grumman, focused on two systems: the Biohazard Detection System (BDS) that the USPS has been using for more than a decade and the Next Generation Automated Detection System (NG-ADS) that the company has developed and field tested under a Generation 3 Phase 1 contract with the Department of Homeland Security (DHS). NG-ADS, Tilles explained, evolved from LLNL's APDS device, which Mariella had discussed earlier.

BDS, which was developed in the wake of the anthrax letter incident in 2001, screens mail as it enters the USPS network and provides a warning before contaminated mail is distributed through the mail network. In a sense, Tilles said, BDS can be considered "Lab-in-a-Box 1.0," an autonomous system that collects samples adjacent to a USPS mail processing machine. Samples are taken over the course of an hour, during which time between 30,000 and 40,000 letters could have been processed. The USPS can track those letters to isolate them in the event of an alarm. At the end of an hour, the sample moves into a reservoir for PCR testing, while the instrument starts collecting the next sample. Sample preparation and PCR are performed using a disposable cartridge operating in a Cepheid GeneXpert machine, and the results are then reported.

So far, the USPS BDS system has conducted more than 11 million tests with no false-positives. Tilles said that the system has been validated by a number of federal agencies outside the USPS, including the Department of Defense and the Centers for Disease Control and Prevention (CDC). The USPS also collaborated with the U.S. Army's Edgewood

Chemical Biological Center to create a test bed to assess system performance under simulated operational conditions. National deployment of the system was completed in 2005, and more than 1,000 detectors have been installed at hundreds of sites. These detectors are networked in a fully integrated support infrastructure that not only transmits data but also monitors the health and status of the instruments; manages consumables, spares, and repairs; and conducts trend analysis for continuous improvement. The National Field Monitoring Center, run by Northrup Grumman, oversees operations of the system.

NG-ADS, Tilles explained, is similar to BDS in that it is an automated system that collects, processes, and analyzes samples. "But because of the requirements of the BioWatch mission, there are some pretty significant differences as well," he said, particularly in terms of assay flexibility. NG-ADS uses a separate fluidic module to perform sample preparation and the Luminex bead array technology to perform multiplexed PCR assays that can detect up to 50 discrete targets from a single sample. Northrop Grumman has built more than 50 units and subjected them to multiple tests at third-party and government laboratories. Tilles characterized NG-ADS as being close to, if not at, TRL 8 (see Table 4-1). As it currently stands, NG-ADS is running 22 signatures, including control signatures, to ensure that there are no non-specific reactions occurring. He added that, guided by experience with the BDS and the Postal Service, NG-ADS has added diagnostic features. In closing, Tilles emphasized the importance of iterative testing, noting that NG-ADS has undergone extensive testing that revealed some issues that the company has since addressed.

Issues in Systems Concepts and Integration

Stevan Jovanovich, cofounder and chief technology officer for IntegenX, Inc., discussed a series of potential problems associated with automating workflow in a way that is reliable and meets the sensitivity needs of the BioWatch program. The hardest part of integrating workflow, he said, is matching the input of one module to the output of another in a way that retains the highest possible sensitivity; to do this, an integrated system must process as much of the initial sample as possible and then use multiplexed sample analysis to avoid the need to split a sample into aliquots.

TABLE 4-1 NG-ADS Key Performance Parameters

Maturity	TRL 7
Operational environment	Indoor/Outdoor (rain, snow, wind, dust, etc.) Temp: –28°C to 50°C Humidity: 5–100%
Agents	Six agents in current assay, expandable to 20 agents or more Capable of detecting DNA, RNA, and protein threats
Sensitivity	100–600 org/m^3 air
P_D / FAR	Probability of detection >95%, FAR better than 1 in 10^9
Size	28" W × 35" D × 70" H
Weight	~600 lbs with on-board consumables
Power	Standard 110/120 VAC, 20A
Communications	Cellular, 802.11x, Ethernet capable; VPN and other features for data security
Operational	>99.5%
Time to result	2 hrs from end of sample collection
Collection interval	Configurable collection periods up to 24 hrs (or longer); compatible with triggered collections

NOTE: FAR = false-alarm rate; P_D = probability of detection; VAC = volts alternating currents; VPN = virtual private network.
SOURCE: Tilles presentation, June 25, 2013.

Using technology developed to process human clinical samples combined with other technology that the company developed, IntegenX has built several integrated sample-to-sequence next-generation systems that produce results in a few hours. The RapidHIT 200 system, for example, uses paramagnetic beads to purify DNA and position it for PCR analysis, processing large volumes of sample into nanoliters of beads. A set of modules makes up the integrated processing system. The current instrument, Jovanovich said, can process from 1 to 8 samples in 70 minutes, and he believes that a well-chosen team of investigators engaging in a 2- to 3-year effort costing about $15 million could produce a set of instruments that would be ready for extensive field testing.

Jovanovich said that in his opinion the most cost-effective approach would be to build a modular system based on current technologies with a consistent set of fluidic interfaces. He added that he believes that BioWatch should have layers of instruments, some of which detect particles and others that perform more extensive and deeper testing, in order to provide flexibility and the ability to respond to new threats. He argued against funding "moon shots" when it comes to biodefense and argued instead for focusing on what is doable based on today's state-of-the-art technology. He suggested that BioWatch should fund the development of three systems through TRL 7.

Discussion

Before opening the discussion to the workshop attendees, session moderator John Vitko reviewed what he saw as the key messages delivered by the five panelists: According to Tilles, there are now several mature technologies—at TRL 7, 8, or even 9 levels—that can meet BioWatch requirements. Northrup made a provocative statement that "detection is done," that other fields have solved the fluidics problem, and that the uniqueness of the BioWatch system lies in the fact that there is human involvement and that otherwise it is really just another analytical instrument. Vitko noted that several of the talks stressed taking a modular approach and also stressed that it is critical to involve public health laboratorians early in the development and testing process for each module. Vitko said that Knight's discussion of pan-genomics and what it means to do genetic testing introduced him to concepts he had not thought about before and raised important questions for the future of detection based on genomics. Vitko was also struck by Knight's clear roadmap for developing multiplexed assays by 2016 and direct sequencing by 2020.

John Plante from the Chicago Transit Authority (CTA) noted that one of the challenges with testing in a transit environment, which is not quite indoors or outdoors, is that the environment changes over the course of a year's time and that instruments will need to be adjusted to accommodate those changes. In addition, the transit environment contains significant levels of "brake dust" that adheres strongly to surfaces and any instrument would have to be able to tolerate it. Tilles remarked that his team experienced that seasonality as well as the brake dust phenomenon when it was testing its system in an urban subway system and that the information they gained from those tests will be valuable going

forward. He reiterated the importance of conducting extensive cycles of testing and design and retesting and redesigning to develop a field-worthy instrument.

Jeffrey Runge said that he appreciated Jovanovich's prescription for how the government might fund next-generation technologies and asked the other participants if they had any additional suggestions. Northrup said that the program needs to be less myopic about technology and that it should bring nondefense companies into the effort, an idea that Paul Schaudies, with GenArraytion, also stressed in his comments to the panel. Tilles echoed Jovanovich's desire to see a more consistent effort that maintains momentum through the long development process that will enable putting together the necessary workforce and allow for the extensive and repeated testing. Jovanovich agreed with Northrup and added that maintaining flexibility and not selecting a winning technology too early in the process would be important to the eventual success of a BioWatch autonomous detector program.

Toby Merlin, director of the Division of Preparedness and Emerging Infection at CDC's National Center for Emerging and Zoonotic Infectious Diseases, asked if any of these technologies could be married to orthogonal technology[2] to confirm positive results. While Mariella said that this was a big challenge and would probably occur on the 2020 time frame, Tilles said that multiple PCR signatures are, in effect, a low-technology approach to orthogonal testing and that designing in flexibility in the fluidics systems will provide the means of integrating orthogonal technologies in next-generation instruments. He also raised the issue that orthogonal detection can increase the possibility of false-negatives while adding substantial cost to the operation of the system. Northrup, agreeing with Tilles, said that orthogonal testing would only be feasible from a cost perspective as a confirmatory test. Knight agreed with that comment.

William Raub asked if next-generation instruments will be able to output self-diagnostic information, and both Mariella and Tilles answered that this capability will be included in Generation 3 and any future instruments. In the same vein, Vitko asked if the instruments would retain an aliquot of sample for retesting, and Tilles answered that this capability is included in next generation instruments under development and testing. Northrup noted that the pharmaceutical production industry

[2]In other words, mutually independent technologies.

has extensive experience developing both of these capabilities and that these should not be viewed as challenges for BioWatch.

Both Sandra Smole and Erica Pan voiced the opinion that public health does not really care what technology is used but that it does want to know how it works and to have confidence in the technology. "We want the technology to work and we want to know that we're going to make good decisions based on that technology," said Pan.

AUTONOMOUS DETECTION SYSTEMS USING IMMUNOASSAYS AND PROTEIN SIGNATURES

To start the second technology session, Paul Schaudies, chief executive officer of GenArraytion, Inc., and author of the commissioned paper on immunoassays and protein signatures (see Appendix H), said that, instead of reviewing his paper, he wanted to make one point. BioWatch, he said, has been the subject of recent critical reports claiming that the system has a high rate of false-positives related to the detection of *Francisella novicida*, a relatively harmless species that occurs naturally in the environment and is closely related to the pathogen *Francisella tularensis*. But in his opinion, he said,

> That is the best real-world data demonstrating that the system works because there was a collection, there was an extraction, there was an amplification, and there was a signal. The piece of DNA that was being looked at was not the right one, but that was not a failure of the system. That is a failure of the test. The fact that the BioWatch program is able to collect organisms that are traveling miles in the air at a very dilute concentration is probably the best data supporting that the system would find something or will find something if it is ever released.

Following those remarks, four panelists made presentations on technologies for detecting pathogens based on protein signatures. The discussions focused on automated multiplexed immunoassays, a disc-based collection and detection system for protein signature determination, a point detection system based on Raman spectroscopy, and a single-molecule array technology for multiplexed detection of proteins and nucleic acids. When he introduced the panel, Schaudies noted that the systems from Luminex, PathSensors, and Battelle are already being tested in the field. The system from Quanterix, he added, is just now being manufactured for eventual field tests.

Automated Multiplexed Immunoassays

Amy Altman, vice president for biodefense and food safety at Luminex Corporation, began by noting that protein signatures can serve as an important detection tool for bacteria and viruses because of the diverse nature of the protein epitopes that cover the surface of these organisms and which can be readily detected individually using antibodies. When used in a multiplexed assay, a panel of antibodies designed to identify the specific combination of protein epitopes that characterize a microbial threat can rapidly and sensitively distinguish between pathogenic and nonpathogenic organisms and strains of organisms.

Immunoassays—the technology that uses antibodies to detect specific protein epitopes—are fast, robust, and efficient, Altman said, and they are able to produce results in 10 minutes to 1 hour, depending on the desired limit of detection. Multiple-epitope analysis provides a high level of specificity that can allow for near-neighbor discrimination, and the ability to detect phenotype versus genotype can provide information on toxicity and viability. Immunoassays can also detect toxins directly, as opposed to detecting the genetic signature of a toxin. Altman said that it would be hard to engineer an organism to escape detection via a multiplexed immunoassay because any alterations that change protein epitopes may render an organism nonvirulent.

Immunoassays are inexpensive to carry out, largely because they are based on a well-developed technology platform and come without the patent licensing burden associated with PCR. Sample preparation is significantly less complicated than for PCR analysis, and immunoassays are less sensitive to dirty samples. In addition, multiplexed immunoassays used for bioweapons detection can be carried out with the same platform that is in widespread use for clinical diagnostics. In fact, Altman said, the system she was highlighting in this presentation for use in Northrop Grumman's NG-ATS is also approved by the FDA for clinical use.

The multiplex technology that Luminex uses, which it calls xMAP, relies on a set of polystyrene microspheres that are color-coded with a unique ratio of a red dye and an infrared dye so that a 10-by-10 matrix of these beads would yield 100 spectrally distinct bead classes, each of which can be linked to a specific antibody for detecting a specific protein epitope. Altman said that in addition to antibodies, the beads can be linked to enzyme substrates, receptor ligands, and nucleic acid probes. Luminex, she added, has recently developed a third dye that will extend the system's

capability to the point that it can multiplex 500 different spectrally distinct beads per assay.

The company's personal computer–sized MAGPIX instrument is capable of performing simultaneous measurement of 50 targets in a single assay at a cost that is one-fourth that of a comparable enzyme-linked immunosorbent assay (ELISA). This modular, FDA-approved instrument can detect single-digit picogram levels of protein as well as 1 million copies of DNA, and it uses LED-based illumination and charge-coupled device (CCD) image capture to improve reliability over older laser-based systems. After describing the mechanics of how the instrument works, Altman said that one of the strengths of a bead-based platform versus a planar array-type technology is the ease of adding new targets as dictated by threat assessments. She added that modern antibody production technology also offers the possibility of improving assay performance by adding or updating assay antibiodies once they are developed in response to a new threat or to better detect current threats.

Altman also addressed the benefits of multisignature analysis. As an example, she reviewed an assay developed with a commercial partner, Radix BioSolutions, to detect multiple epitopes of the toxin ricin. This 18-plex assay accounts for natural or intentional genetic drift and extends the dynamic range of the assay. It also decreases the false-alarm rate using decision theory software to analyze assay data.

In her final remarks, she said that the MAGPIX module has been integrated into Northrop Grumman's NG-ADS unit and has been tested extensively by third parties. What remains is for the device to undergo field testing to determine its real-world performance in an environmental test setting. She said that the company believes that the technology could be TRL 6-plus ready by 2016 if it can demonstrate the same immunoassay performance in an autonomous system that it does on the benchtop and if it is confirmed that stable reagents can be deployed and provide stable performance over the entire service period. She also noted that while immunoassays may not have the same level of sensitivity as a PCR-based assay, it is important to think in terms of clinical relevance, and the sensitivity of protein signature detection may be sufficient for the purposes of biothreat detection.

Rapid Detection of Pathogens in Aerosol Samples

Originally developed at Massachusetts Institute of Technology's Lincoln Laboratories (Rider et al., 2003) and now being further developed at

PathSensors, the technology uses pathogen-specific antibodies to detect pathogens and toxins collected using the company's proprietary aerosol collection technology. According to Ted Olsen, chief executive officer and president of PathSensors, Inc., the system can provide pathogen and toxin detection in less than 5 minutes once the sample has been collected in its disc-based collector and analyzer. The technology is called CANARY, short for Cellular Analysis and Notification of Antigen Risks and Yields, and it detects bacteria, viruses, and protein toxins simultaneously. It uses a genetically engineered B-cell lymphocyte that, in response to protein binding, releases a calcium spike internal to the cell that in turn triggers light emission that can be detected outside the cell. An instrument based on this technology, Olsen said, is already used for mail screening in smaller mailrooms that do not opt to use the USPS system designed for higher volumes.

When loaded into the BIO-FLASH instrument, the center of the compact disc–sized collector sits in an airstream and, as it spins, collects air in 1 of 16 radial channels, each fitted with a light sensor. Sample collection can run from 20 seconds to hours. Once the sample is collected by inertial impact on the wall of the disc, the disc continues to spin as reagents are released into the channels. Almost as soon as the reagents come in contact with the sample, light emission occurs if the target organism or protein is present, triggering a visual and audible alarm. Olsen explained that the device can be configured to assay for the same pathogen multiple times in the disc, run positive and negative controls, and retain a sample for later recovery and analysis. The instrument also has the ability to upload data to a remote location.

Olsen noted in closing that the instrument has undergone extensive third-party validation. Those tests have shown that the system is capable of identifying with 100 percent accuracy the biological agents in aerosol samples containing between 75 and 450 agent-containing aerosol particles per liter of air, which he said meets or exceeds the targets set by the BioWatch program.

Detecting Biothreats with Raman Spectroscopy

Andrew Bartko, principal scientist at Battelle Memorial Institute, said that the Resource Effective Bio-Identification System (REBS) that Battelle Memorial Institute has been developing collects and identifies bacteria, viruses, and toxins directly from atmospheric aerosols in a nondestructive manner that is compatible with genetic confirmation. In field tests, the REBS system has demonstrated sensitivity below the lower limit set by the

BioWatch program and has produced no false-positives in actual environmental testing. The system, which can be updated to accommodate new threats in 24 hours, collects continuously and stores an environmental particulate history that can be accessed and reexamined if necessary. Bartko said that REBS is not meant to provide an end result but rather to act as an alert signal that will require further confirmation using other methods. The cost per sample analysis is less than 1 cent, and each portable, battery-powered instrument costs less than $100,000.

The core technology in REBS is Raman spectroscopy, a method that measures the unique vibrational frequency of molecular bonds. Because of the large number of bonds in a biological material, the resulting spectra are complex, and Bartko and his colleagues have been studying the Raman signals intensively to understand their nuances in order to use them for species-level identification. REBS is now able to identify mixtures of different types of threats and report how many of each type of cell is in a given sample. "In some instances," he added, "we have evidence that the cell viability can be indicated by looking at specific features within the spectral features." Though not currently included in REBS, the spectra could distinguish organisms intentionally grown in laboratory media from those grown in natural nutrients. The bottom line, Bartko said, is that REBS can identify biothreat species with no primers, probes, liquid reagents, or substrates; it is capable of being accurate, agile, and economical to run.

The working part of the device consists of an inlet stack, an electrostatic collector, a metalized polymer tape in a cassette for collecting samples, and the optics system. Particles collected on the tape are illuminated by a laser, producing the Raman spectra. The detector quickly scans the spectra for identifying characteristics, and if those characteristics are spotted, the sample is irradiated further to increase the signal-to-noise ratio and to enable a more detailed examination of the fine structure of the spectra. Identification of a single particle does not trigger an alarm, but it does increase the probability of identifying subsequent particles that the instrument searches for specifically and correlates to the first particle and the spectral library. The REBS instrument can also detect chemical weapons, Bartko noted, adding that tests conducted using simulated releases in a variety of environments, including the Boston subway system, have produced no false-positives and 100 percent identification of the released simulant (see Table 4-2).

Looking to the future, Bartko said that REBS is currently at TRL 6, with additional operational tests and evaluation still needed to demonstrate that the system meets or exceeds 99.9 percent reliability. Field tests have

demonstrated zero false-positives with a 95 percent confidence, but further operational tests are needed to demonstrate a lower false-positive rate, and further work is needed to integrate REBS with other technologies that can confirm the REBS signal.

Single-Molecule Array Technology

In contrast to the other technologies described in this session, Quanterix's single-molecule array (SIMOA) system (Rissen et al., 2010) has only been developed for use in human diagnostics and has not yet been applied to the detection of potential biological threat organisms in the environment. As David Hanlon, director of business development and strategic collaboration at Quanterix, described the technology, its digital nature enables single-molecule resolution. What makes single-molecule detection possible, he said, is the ability to trap individual molecules in femtoliter-sized wells, which are some 2 billion times smaller than a standard ELISA well. The miniscule volume allows for a rapid buildup of fluorescent signal that is readily visible.

Like other protein detection schemes, this one uses antibody capture reagents attached to paramagnetic beads. In this case, the beads are sized so that only one bead will fit in a femtoliter well, and a large excess of beads are used so that the beads will bind to at most one target molecule. After the capture beads are allowed to bind with proteins in blood, serum, or other biological matrices, the mixture is flowed over the wells, detection reagent is added, and the wells are sealed with oil. If the target analyte has been captured, the capturing well will fluoresce. As currently designed, this technology can multiplex up to 10 analytes.

Tests on clinical samples for a wide range of substrates demonstrate that this technology routinely achieves sensitivities in the femtogram-per-milliliter range, compared with the pictogram-per-milliliter limit of detection for other technologies. In one test, for example, SIMOA was as sensitive as PCR for detecting the human immunodeficiency virus prior to seroconversion. Hanlon noted that with this increased sensitivity, it should be possible to detect the vast number of proteins with potential relevance to human disease that are now beyond the reach of current detection technologies. The company has also recently demonstrated that it can detect DNA without amplification in complex samples such as blood and river water. Quanterix has partnered with a manufacturing company, Hanlon said, and it plans to introduce an instrument for research use in late 2013.

TABLE 4-2 Summary of Government Testing of REBS

Location (Sponsor)	Date	Type	Result
Fort Bliss, Texas (JPM-G)	6/2009 to 7/2009	Background sampling	Zero false-positives
Dugway, Utah Piggyback (JPM-BD)	10/2009 to 11/2009	TRE simulant challenge in realistic environment	ID sensitivity—25 ACPLA
Battelle Columbus, Ohio (JPM-BD)	11/2009 to 01/2010	JBTDS identifier TRE samples, ID performance with killed BWA	100% identification
Battelle Columbus, Ohio (JPM-CA)	6/2009	CWA (particle) ID feasibility	Dual-threat feasibility proven
JHU-APL/ECBC (JPM-BD)	9/2010	JBTDS TRE shoot-off with simulants	Selected for JBTDS competitive prototyping
DPG (JPM-CA for CBDS)	2/2011 to 7/2011	ASEC and ABT testing with irradiated materials, unknown biological, near-neighbors, and Interferants	Demonstrated sensitivity, specificity, and false-alarm performance
Boston, Massachusetts, Subway Test (DHS S&T)	10/2012 to present	Simulant release in actual environment	Zero false-positives, 100% identification of simulant release
West Jefferson, Ohio (CRADA w/JPM-NBC)	06/2013 to 07/2013	Live agent integrated system testing and ABT	TBD

NOTE: ABT = ambient breeze tunnel; ACPLA = agent containing particles per liter of air; ASEC = aerosol simulant exposure chamber; BWA = biological warfare agent; CBDS = Chemical and Biological Defense; CRADA = cooperative research and development agreement; CWA = chemical warfare agent; DHS S&T = Department of Homeland Security Science and Technology Directorate; DPG = Dugway Proving Ground; ECBC = Edgewood Chemical Biological Center; ID = identification; JBTDS = Joint Biological Tactical Detection Systems; JHU-APL = The Johns Hopkins University Applied Physics Laboratory; JPM-BD = Joint Program Manger Biological Defense; JPM-CA = Joint Program Manager Contamination Avoidance; JPM-G = Joint Program Manager Guardian; JPM-NBC = Joint Program Manager Nuclear Biological and Chemical; TRE = technology readiness evaluation.
SOURCE: Bartko presentation, June 26, 2013.

Discussion

Schaudies remarked that one approach to improving the sensitivity of antibody-based technologies without increasing the cost or the time that it takes to conduct the test would be to sonicate a sample using existing technology. What this would do, he explained, is disrupt the cell membrane of the viral coat and create thousands of liposomes, each with one or a few epitopes on it rather than one entity—the original cell or virus particle—with thousands of epitopes. Such an approach, he suggested, could produce sensitivities equivalent to nucleic acid technology with the speed of antibody technology. He then challenged DHS to look beyond the pathway that it has been on. He cited the presentations made in this session as examples of new ways of thinking about the technologies that will enable BioWatch to create the next generation of autonomous instruments. "I can make arguments for each one of these technologies to be in a system," he said.

Bartko was asked by several workshop participants if Raman spectroscopy would work in environments where the biological background was high. He replied that the system can do that now thanks to years of studying Raman spectra from biological samples and learning which features are the most important to look at for the organisms of interest. This information makes up an important part of the software that the instrument uses to determine which particles to investigate for longer periods of time.

Panelists agreed with the comments from several participants that these technologies are not meant to replace nucleic acid–based tests but rather to augment them as part of an orthogonal test paradigm. There was also agreement that different systems may be more appropriate for different testing environments or for testing over different time frames. Schaudies emphasized these points, noting that hours could be saved if immunoassay or Raman approaches were used to rapidly detect a potential biothreat and trigger the more sensitive, specific, and costly nucleic acid–based detection. In a final comment, Jeffery Runge noted that attribution is important and that any autonomous detection system must have archiving capabilities. The panelists agreed and responded that their systems all have the ability to store samples for subsequent analysis.

AUTONOMOUS DETECTION SYSTEMS USING GENOMIC SEQUENCING

In introducing the panel for the third technology session, Rita Colwell, Distinguished University Professor at the University of Maryland and the Johns Hopkins University Bloomberg School of Public Health, commented that the previous sessions had made a strong case for the proposition that it is necessary to have a multidimensional view of identification. "Each of these approaches—and we'll hear about the very powerful one of sequencing today—provides a dimensional understanding and contributes to accurate identification of microorganisms, which is really what we're about," she said.

Colwell also said that she believes that the BioWatch program should shift its perspective, partner with the National Institutes of Health (NIH), and bring public health laboratorians into the discussion. She noted that while BioWatch is focused on detecting the worst pathogens, the tools that the program is developing are essentially the same tools that the biomedical community is developing to rapidly diagnose human diseases. The BioWatch community should be building on all of the information that NIH has developed regarding infectious organisms and genomics, she said, particularly with regard to the issue that Ivor Knight raised in his talk about microbial pan-genomes.

For this session, the commissioned paper was written by Chris Detter, director of the Bio-Threat/Bio-Defense Program in the Emerging Threats Program Office of the Global Security and Bioscience Division of Los Alamos National Laboratory (LANL), and by Gary Resnick, an independent consultant and guest scientist at LANL (see Appendix I). Panelists reviewed the current state of sequencing technologies and provided a glimpse of the future of genomic sequencing; discussed informatics issues involved in developing useful information from advanced sequencing technologies; and described real-world applications of next-generation sequencing and how those might integrate with the type of sampling methods already used in BioWatch.

Current and Future State of Genomic Sequencing

Chris Detter said that the focus of the commissioned paper was BioWatch's three primary needs: that any system provide affordable and continuous coverage of at-risk populations, that it have accuracy and precision to effectively support potentially high-regret responses, and that it be

responsive to the full scope of biothreats. But before addressing those three needs, he briefly reviewed the history of modern sequencing. It started in 1975 with Sanger sequencing, he said, preceded through the era of "automated" gel sequencing, and then entered the era of the Human Genome Project and its use of Applied Biosystems capillary sequencing, starting in 1999. During the past decade and a half, a variety of technologies have produced a dramatic drop in the cost of sequencing and a dramatic increase in its speed, and some of the newest technologies can perform single-molecule sequencing, which does not require amplification. Each technology, Detter said, has its own set of error rates and error issues and is suitable for a particular set of applications.

The workflow for so-called next-generation sequencing is fairly uniform for both clinical and environmental samples, consisting of a set of preparatory steps followed by sequencing and then a computational analysis of the sequencing reads to assemble the entire genome (see Figure 4-4).

Detter discussed three applications of next-generation sequencing. The first was rapid sequencing of the amplicons from PCR reactions to identify and characterize pathogens; this amplicon sequencing can readily test hundreds of samples. Shotgun sequencing of multiplexed environmental or clinical samples can identify and characterize both known and emerging pathogens. And full-genome sequencing of a single organism, after the organism is isolated and grown in the laboratory, can identify sequences that are associated with specific outbreaks and can rapidly detect the same pathogen in future outbreaks.

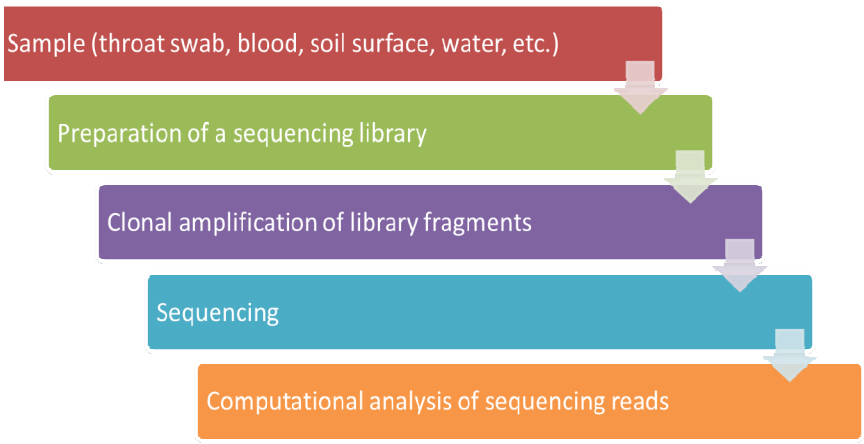

FIGURE 4-4 Next-generation sequencing workflow.
SOURCE: Detter presentation, June 26, 2013.

In the commissioned paper prepared for the workshop, Detter and Resnick concluded that high-throughput amplicon sequencing could be at TRL 6 by 2016, with some engineering and integration work to fit it into the BioWatch system. Amplicon sequencing would have limited ability to identify emerging threats, particularly RNA viruses, but it should be able to detect all known pathogens. By 2020, improved next-generation sequencing technologies, in combination with bioinformatics, should enable metagenomic sequencing of entire environmental samples at TRL 6. These systems would be capable of detecting emerging and engineered pathogens. The main limitation will be the need to prepare sequencing libraries, which will limit the speed of these processes. Beyond 2020, cutting-edge sequencing technologies that do not require amplification should be at TRL 6. They will provide the same capabilities as those that should be ready by 2020, but on a much faster time scale.

In his final remarks, Detter noted that sequencing is rapidly becoming the gold standard for biological identification and is causing biologists to revisit the concept of phylogeny in microorganisms. Today, sequencing can cover the entire threat, known and novel; two examples are the discovery of SARS (severe acute respiratory syndrome) and the discovery of the MERS-CoV (Middle East respiratory syndrome coronavirus), both of which were done through sequencing. "We still don't know the full potential of sequencing for biodetection in the field," Detter said, but the hardware and knowledge bases that are available and that are being improved daily are designed from the start to be used in high-throughput applications. He sees these technologies as being dual-use platforms that can share the cost, risk, and actual processes across fields, particularly between forensics and biosurveillance, which share similar adoption paths and processes.

Next-generation sequencing has already transformed microbiology and can be expected to transform it even further with NIH's human microbiome project, the Beijing Genome Institute's 10,000-microbial-genome program, and the Joint Genome Institute's microbial earth project, Detter noted. All of these efforts will generate a tremendous amount of sequence data about the microbial world that BioWatch should put to use as it develops and adopts its own sequencing technologies for biodetection, Detter said. Bioinformatics and metadata will play a critical role in making use of these databases, and Detter encouraged the community to share knowledge bases and bioinformatics capabilities. Another key to the successful adoption of sequencing technologies will be the development of autonomous and modular platforms that can be integrated into the

BioWatch system. "Ease of use is going to be critical for wide acceptance," he said. For any system to be adopted, "it must be responsive to public health officials' needs and provide acceptable and actionable information for their decision-making process."

Challenges and Opportunities in Genome Sequencing Technologies

Jeffrey Schloss, director of the Division of Genome Sciences and program director of Technology Development and Coordination at the National Human Genome Research Institute (NHGRI), began by noting that most of the next-generation sequencing technologies were developed as a result of the NHGRI's realization toward the end of the Human Genome Project that sequencing the human genome was only the first step toward realizing the full potential of genomics, not just for the study of human diseases but also in many fields, such as agriculture and microbial ecology. Schloss stressed that much of the increase in sequencing speed has come from improving the workflow in going from Sanger technologies to current next-generation sequencing technologies. Gone are the days of having to clone every fragment of DNA in a bacterium, then picking a suitable colony and purifying its DNA for sequencing. Instead, all of that processing is now done in tubes, and while still technically challenging, this new workflow is easier. The hope, he added, is that this process will become even easier with further technology development. The end result of the past decade of development, Schloss said, is that in 10 years sequencing output has gone from 100-base pair reads per sequencing read to more than 100 million reads per run, and sequencing has gone from the province of centralized sequencing centers to individual laboratories. Today, there are sequencing machines the size of a personal computer.

NHGRI has not supported much research on sample preparation, Schloss said, but the private sector has, and there are now companies that are commercializing integrated sample preparation and library preparation. These types of integrated systems will be important in any deployable system, such as those envisioned for BioWatch, as well for moving sequencing from the research laboratory to the clinical setting.

Unlike earlier DNA sequencing technologies, next-generation methods sample very large numbers of single molecules or ensembles generated from single molecules, and this approach enables new biological insights. For example, Schloss said, researchers are now able to look for sequence variations, rare variants, and haplotypes, which he said will be important to

BioWatch because it will allow sequencing to identify which bacterium in an environmental mixture has an antibiotic resistance gene. The important point, he said, is that these techniques are digital, enabling the detection of rare signals in a mixture. The question of exactly how rare remains to be answered.

By the end of the Human Genome Project in 2003, sequencing 1 human genome took 3 months with 100 capillary array electrophoresis machines working together. By 2009, one machine using sequencing by synthesis on array technology could sequence a human genome in less than 1 month. By 2011, one machine could sequence three genomes simultaneously in 1 week. And commercial instruments released in the past year reportedly can sequence a human genome in just 24 hours; Schloss said he has not seen those claims verified in practice yet, but they do give an idea of what the future holds in terms of sequencing speed.

The other important trend in sequencing has been the precipitous drop in the cost of sequencing, particularly since 2008, when prices began plummeting. Schloss noted, however, that the cost per genome appears to be leveling off, and he said that he suspects that one reason is that the field seems to be converging on a single technology and an accompanying reduction in competition.

Turning to what lies ahead for sequencing, Schloss discussed several technologies now being developed. Free-running polymerase technology, for example, could provide real-time DNA sequencing from single polymerase molecules using zero-mode fluorescence detection (Eid et al., 2009). This technology demonstrated read lengths of almost 3,000 bases in 2011, and it is reported to be improving rapidly. Because it sequences directly off genomic DNA and does not require library preparation, it can be used to identify methylated or damaged bases that might be of interest (Flusberg et al., 2010). Other new technologies can sequence RNA directly using reverse transcriptase (Vilfan, 2013), and these methods are already identifying novel modifications in the RNA of microbes and are allowing researchers to map and detect these modifications at the single-base level, enabling new biology.

An exciting development in sequencing is the use of nanopores to sequence DNA in real time without the use of enzymes (Clarke et al., 2009; Kasianowicz et al., 1996; Stoddart et al., 2009). Instead, Schloss explained, nanopores detect changes in ion flow through the pore as a single molecule of DNA moves through the pore. He noted that this type of technology would simplify the MicrobioMe project by enabling the rapid, nondestructive sequencing of DNA—and potentially RNA—on a massive scale.

Turning nanopores from an interesting research idea into a practical sequencing method has been incredibly challenging, but progress has accelerated over the past few years to the point that investigators are starting to generate data that resemble sequencing reads from nanopore devices (Ayub and Bayley, 2012). It may also be possible to use nanopores to detect proteins and distinguish among different proteins (Soskine et al., 2012).

Next-Generation Sequencing of Microbial Populations

Predicting the future impact of next-generation sequencing is a daunting challenge given the pace of technology development, said Thomas Cebula, chief technical officer at CosmosID and visiting professor at Johns Hopkins University, but he felt comfortable with the idea that as more people use these technologies they will develop an increasing number of applications. As an example, he discussed how researchers in cancer biology are now looking to next-generation sequencing as a technology that will change how patients are treated in the near future (Kamalakaran et al., 2013). "This is the future," Cebula said. "The question is, *When* is the future?"

Though the cost of sequencing has fallen significantly in the past decade, a major part of the expense arises from the need to provide multiple coverage—on the order of 30-fold—in order to be able to assemble a genome from the short reads generated by next-generation sequencing technologies. "But what if you didn't have to assemble the genome to make a call of what's in a sample?" Cebula asked. "That would cut our costs and bring the time frame forward for doing real-time detection." It would also provide the ability to interrogate the entire microbial genome rather than use representative gene markers, as is currently done, to characterize microbial genomes.

The approach that CosmosID is taking is to develop algorithms that take unassembled reads from a metagenomic microbial sample and return, within minutes, identification at or near the strain level. He noted that there are other software packages available that make identifications at the species level. In his opinion, strain identification is important for forensic purposes because it can provide source identification and attribution. In support of this contention, he showed how the CosmosID software package can identify and distinguish between strains of *Escherichia coli* O157:H7, only one of which was responsible for outbreaks of human illness.

Metagenomic Sequencing in Biodetection

In his presentation, Brian Young, program manager at Battelle Memorial Institute, addressed the question, "Why consider metagenomic sequencing in autonomous identification?" The major reason, he said, is that it is a single method that can identify a broad spectrum of biothreat agents and that can meet all three levels of the current PCR strategy: primary screening, verification, and subspecies identification. Metagenomic sequencing can also identify unexpected species, provide an alert when an unknown species is present, and monitor the environmental background and normal variation in that background while searching for target species (see Table 4-3). This type of background monitoring can provide evidence to help determine whether a potential threat organism, such as *Francisella*, is present because of an intentional release or because of an environmental disturbance. In the case of the latter, the prevalence of other organisms is likely to undergo coincident increases or decreases in response to the disturbance.

Metagenomics, Young added, can also provide deep genetic evidence for use in identifying the strain or even the type of the agent, including the identification of virulence and antibiotic resistance. Nucleotide-level discrimination may also be useful for forensic attributions. As an example, Young cited work done at Battelle in which morphologically different colonies of *Brucella suis* were sequenced, revealing single-nucleotide variations that could be used to identify the different colonies. He echoed Cebula's comments that software and bioinformatics packages will be key to developing these types of applications for rapid identification purposes.

On the subject of how metagenomic sequencing would fit into an autonomous detection system, Young said that it would be similar to the approach used with PCR. The main issues, he said, would be systems integration and testing the integrated systems. The main difference from current PCR systems would be the need to perform data analysis locally because of the time that would be required to transmit the raw datasets to a central processing location. Performing the analysis locally will require the development of computationally efficient software, which Young said will be a challenge. Another challenge to incorporating next-generation sequencing into BioWatch is that sample preparation must be better automated and mated with air sampling systems. At present, most workflows are not deterministic or diagnostic, which is what BioWatch requires. Operational field sensitivities have not yet been established, nor have opera-

TABLE 4-3 Monitoring Abundances of Target Environmental Organisms in Oil-Exposed Sediment Using Next-Generation Sequencing

Organism	Relation to Oil Degradation	Percent of Bacterial Sequences Found With and Without (W/O) Exposure to Oil			
		Rhode Island		Alaska	
		W/O	With	W/O	With
***Pseudoalteromonas* spp.**	Aerobic gammaproteobacteria; several species considered oil degraders	0.10%	13%	1%	9%
***Alcanivorax* spp.**	Aerobic gammaprotebacteria; principal carbon source is linear-chain alkanes	0.40%	2%	0.07%	0.20%
Escherichia coli	Facultative anaerobic gammaproteobacteria; not associated with oil degradation	4%	0.20%	0.60%	0.80%

SOURCE: Young presentation, June 26, 2013.

tional field specificities or false-positive rates. Furthermore, the cost of sequencers and sequencing consumables are still too high for routine use. The DNA inputs required for sequences are an order of magnitude too high for practical field use without amplification, and adding amplification would essentially turn these instruments into high-end PCR detectors. Metagenomics could prove to be disadvantageous in high-background scenarios in which heavy pollen or mold spore loads would consume sequencing bandwidth and reduce sensitivity to pathogens. Developing techniques for removing eukaryotic DNA background could solve this problem, Young noted.

In terms of technology readiness, Young estimated that the integration of aerosol collection with next-generation sequencing, autonomous operation of sample preparation, and autonomous operation of sequencing are "TRL low." Data analytics, he estimated, are actually more developed and at TRL 7, and he showed results from tests using the CosmosID system as an example of how far data analytics has come over the past few years. In closing, Young said, "What I'd like to leave the audience with is that no matter what technology gets deployed in [autonomous detection], genomics sequencing has to be in the equation somewhere. You're going to

want it in the lab to do the attribution analysis; you are going to want it in your system for the interrogation that ultimately will happen." As a result, he said, any technology that gets deployed should at the very least preserve a sample or not destroy the sample so that it can be sent to the laboratory for sequencing.

Discussion

John Vitko asked if any of these technologies can meet the BioWatch goal of reporting within a few hours of sampling. Detter replied that Tier 1 technologies will have a turnaround time of about 10 hours after sample collection is complete. Tier 2 technologies should reduce that to about 6 hours, and Tier 3 should drop that further to below 4 hours. The big unknown, he said, is sample preparation time.

Cindy Bruckner-Lea, from the Pacific Northwest National Laboratory, asked what sequencing provides in the proposed Tier 1 device (sequencing of PCR products) that PCR-based detection does not, given the extra cost and time of sequencing the PCR products. Detter said that instead of merely getting a band (indicative of presence/absence of a PCR product), sequencing identifies the one or more components of that band. He acknowledged that it may not be necessary to do this in the field or as the primary application in Tier 1 devices but rather as a confirmatory test.

In response to a question from Colwell about the quality of sequence data in public databases, Detter said that there is an effort under way to create what he calls a trusted database. This database would have quality scores associated with the data, and sequences would be checked against near neighbors to generate a set of curated pathogen sequences. The diagnostics community is leading this effort.

Thomas Companion from the Office of Standards in the Science and Technology Directorate at DHS asked how next-generation sequencing technologies will deal with the various structural variations of DNA that are found in organisms and with genome dynamics, such as in the case of organisms that edit their DNA as they use it. Detter replied that he does not see those as issues that are beyond solving with well-curated databases to a level that will be satisfactory for BioWatch purposes. Cebula agreed with that assessment. Schloss added the issues of variability and change are also being addressed by those who are studying complex human diseases. He said that as the research community builds its databases, it will be possible to identify the normal variation seen in environmental samples.

Raymond Mariella, Jr., asked about the problem of contamination resulting from carryover from one run to the next. Detter replied that today's sequencers use disposable slides or chips, which solves what was an issue with older capillary sequencers. Schloss said that this issue is being addressed by the clinical community because it has the same concerns about carryover in sample preparation and sequencing.

Mariella then asked why there seems to be so little work on viruses, which can also be important biothreat agents. Cebula said that part of the reason is that the taxonomy of viruses is still poorly understood, making it harder to correlate characterization work with a particular species or strain. Detter replied that another advantage of metagenomics is that it will allow for more efficient investigation of viruses using the DNA and RNA sequencing technologies that he and Schloss described. He added that the Defense Threat Reduction Agency is now working to expand the reference database of viral sequences. Stephen Morse from Columbia University said that while virologists may argue about the fine points of virus classification, the known viral threat agents can be identified today. He said that he also believes that some of the new technologies offer great potential for better understanding viruses.

Allen Northrup asked if BioWatch would be able to afford next-generation sequencing, given the prospects that full deployment would require several hundred million PCR reactions per year. Schloss replied that part of the motivation for using solid-state nanopores is that they would be manufacturable using industry-standard complementary metal–oxide–semiconductor (CMOS) technology that would drive down the cost significantly, although he acknowledged that the effort is still in its infancy. Young agreed that costs must drop and was of the opinion that further development of new reagentless technologies would significantly affect cost. He added, though, that he found it hard to imagine a robust BioWatch program that does not use sequencing somewhere in the process, a comment that Detter seconded.

Cindy Bruckner-Lea commented that using sequencing in the laboratory to confirm results from the field makes a great deal of sense. She then asked the panel to comment on the major challenges in sample preparation. Cebula replied that work still remains to be done to develop a reproducible and automated procedure, but that PCR had the same issues to deal with before it could be approved for routine clinical use. Young said that what is really needed is a program to field test various methods to see what happens with environmental samples. "You don't know what inhibitors you're going to see, you don't know when you're looking at minor components in

a complex background, you don't know how hard or easy it's going to be to detect that until you get out in field testing," he said.

Thomas Slezak from LLNL asked what the BAR of the future will be in the context of next-generation sequencing, given how difficult it was to agree on a definition with PCR. Young replied that the BAR problem for sequencing will be two-dimensional. One dimension will be the depth of evidence or the number of reads needed, and that is an issue shared with PCR. The second dimension is how far across the genome the analysis will have to proceed before the evidence is convincing enough to declare a BAR. Detter noted that the BAR will not be declared in a vacuum and that field results can be confirmed in the laboratory.

AUTONOMOUS DETECTION SYSTEMS USING MASS SPECTROMETRY

For the final technology session, A. Peter Snyder and Rabih Jabbour prepared the commissioned paper (see Appendix J). The panelists discussed, quality issues that are critical for mass spectrometry, the use of single-particle aerosol mass spectrometry (SPAMS) for biothreat detection, and the use of a miniature mass spectrometry system for analyzing microorganisms.

Current State of Mass Spectrometry

Peter Snyder began by explaining that the fundamental principle of all mass spectrometry methods is that they separate gas-phase ions according to their mass-to-charge ratio. There are many methods for ionizing samples and a variety of analyzers for moving the ions from the region where they are created to a detector, where they produce an amplified signal. Mass spectrometry is capable of detecting substances at the low-attomole level, Snyder said, and it generates huge datasets in a short period of time.

On a Tier 1 time frame, there are two mass spectrometry systems that are mature enough to identify organisms based on their structural composition and that meet the DHS criteria: the Hamilton Sundstrand chemical biological mass spectrometer (HSMS) and the SPAMS. The HSMS system, which measures the fatty acid content of a microbial sample, has been refined and tested extensively over the past 20 years, but tests have yet to generate quantitative figures of merit for false-positives and the

probability of false-positives. Doing so, said Snyder, will merely require a larger body of experiments that will take time to complete.

The SPAMS instrument is an improvement on the bioaerosol mass spectrometer developed at LLNL, Snyder said. It uses multivariate data analysis that generates a huge dataset of both positive and negative ions from sampled particles after a preliminary ultraviolet fluorescence analysis conducted as particles enter the system. Because of this prescreen, the system can handle 10,000 particles per second. SPAMS was tested at an international airport and produced no false-positives in a 7-week period, during which time it tracked approximately 1 million particles. The downside to this technology is that the mass spectrometer is large and expensive; however, operating costs are low and a comprehensive set of performance data has generated figures of merit for prediction of false-positives and false-negatives.

Tier 2 systems, Snyder said, cannot begin to address the DHS analytical figures of merit. Though there are many mass spectrometers and sample processing technologies that have the potential to meet those figures of merit, none of the current systems are at the point that they are ready for field testing, and all of them will require appropriate engineering to create a robust, autonomous system. Various microfluidic systems, for example, can concentrate pathogens into a relatively small volume of liquid at a potential operating cost that would be suitable for a large-scale deployment in the field. He noted that biochips have been manufactured to include microcolumn liquid chromatography and electrospray ionization and that work is being done on a microfluidic proteomic reactor that can digest a protein sample efficiently to produce a sample suitable for analysis by mass spectrometry.

In a protein-based system, the peptides resulting from a bacterial protein digest would be analyzed by mass spectrometry and the output would be matched to a complete theoretical list of peptides in a bacterial database generated from genomic sequencing data. The U.S. Army Edgewood Chemical Biological Center has been developing this type of technology for its ABOID (Agents of Biological Origin ID) system and has shown that it can identify specific bacteria in a mixture using mass spectrometry proteomics data generated in the laboratory. Snyder noted that this approach can identify bacteria at the genus level when a specific species is not in the database.

The advantages of proteomics-based mass spectrometry as a biothreat detector are that no prior information about the sample is required for analysis, Snyder said. In addition, no specific reagents are

needed in the analytical process, and it can classify an organism when a primer or probe set is not available. Proteomics mass spectrometry, he added, can provide a presumptive identification of a true unknown organism by mapping its phylogenetic relationship with other known pathogens (Jabbour et al., 2010).

He concluded his summary of the field by stating that there is no system yet envisioned for Tier 3. He voiced the opinion that PCR or an antigen-antibody technology could be interfaced with mass spectrometry to fill this gap in the market.

Critical Quality Issues

Rudolph Johnson, acting manager, Emergency Response Branch, at CDC's National Center for Environmental Health, began by saying that from his perspective as someone who is interested in technology that can be put in the field, the two areas of focus for microbial mass spectrometry are to develop fingerprint spectra for bacteria and to produce interpretable results on a representative number of samples as quickly as possible. Both of these areas are being tackled by the clinical microbiology community, he said, and that community has the money to conduct the necessary research. According to Johnson, the BioWatch community needs to be more concerned about quality in the field setting than in the laboratory, using environmental samples rather than clinical samples.

One of the advantages mass spectrometry has over some of the other technologies that have been discussed is that it is both mature and robust. Mass spectrometry itself is about a century old, and most instrumentation is conserved between applications, Johnson said. Mass spectrometry is a large field, and most of the research effort is aimed at improving the sample preparation, ionization methods, and database analysis, or at developing new applications for the existing detectors, rather than new mass spectrometers. "If you are looking for a new testing platform, you need to consider mass spectrometry," Johnson said.

He said that mass spectrometry is portable, although he noted that "portable" does not mean "stand-alone," as mass spectrometry is usually done with human input. One unusual feature of mass spectrometry is that a smaller sample usually produces better sensitivity and specificity. Sample quality is important, then, to the veracity of the data from a mass spectrometer, particularly in terms of competition for ions during the ionization stage. Johnson offered a caveat regarding the selection of instru-

ments, specifically concerning low- versus high-resolution systems. "The one thing I want to tell you about mass spectrometry is that better is the enemy of good enough," he said. "If you are looking at mass spectrometry, look for something simple." Lower-resolution systems, he explained, are more flexible, less expensive to purchase and to operate, and more reliable.

Data processing is an area in which advances have brought big changes in the field, and Johnson stressed that the goal for BioWatch should not be data interpretation but rather fingerprint identification, a much simpler task. He said that bacterial databases are now being used in hospitals for routine diagnosis, which suggests that these databases have reached a level of maturity and reliability that bodes well for the biodetection field. He also remarked that these databases are transferable between instruments, which is an important development.

In closing, Johnson said that mass spectrometry has reached a stage where it is largely a black box for the average user. However, it is still important to understand the workings of these instruments and their key quality issues. Otherwise, he said, "you will end up with poor-quality data, and the system that you deploy in the field will not be effective."

The questions that every user needs to answer include

- How do you verify instrument performance daily?
- What are your positive and negative controls?
- How do you characterize fingerprint spectra, and who controls database entries?
- What is a minimum threshold criteria for a positive result?
- What is your matrix? Instruments placed in different locations will have different matrices.
- How do you compensate for charge competition?

Single-Particle Aerosol Mass Spectrometry

Eric Gard, scientist in the Defense Biology Division at LLNL, began by saying that one of the advantages of SPAMS is that it eliminates the need to process bulk samples of aerosols, a process that adds sample preparation delays into the analysis pipeline. Instead, aerosol particles are pulled directly from the atmosphere and analyzed independently in a time that is of the order of milliseconds. This short time frame allows for multiple measurements that can improve detection sensitivity and detection statistics (Steele et al., 2008).

The collector for this device is an aerodynamic lens that focuses particles and directs them past tracking lasers that provide information on size, speed, and location of particles as they move through the system. A fluorescent stage excites the particles and measures fluorescence emissions as an indicator of whether a particle is likely to be biological. Based on measurements of a particle's speed, the instrument knows when a prescreened particle reaches the center of the ion source, and at that point it delivers a high-powered laser pulse that produces both positive and negative ions that are then analyzed by the mass spectrometer.

Gard explained that he and his colleagues had what he characterized as a fantastic opportunity over 3 years as different sponsors allowed them to put their instrument through a variety of field tests. As Snyder mentioned, the system was first deployed at an international airport for approximately 7 weeks to test background monitoring in a realistic environment. The second field deployment, which occurred at Fort Irwin, evaluated how it would perform in a dusty environment combined with heavy equipment emissions. Finally, the Applied Physics Laboratory at Johns Hopkins University conducted independent, laboratory-based simulation tests in the presence of high concentrations of highly interfering backgrounds. Over the course of running 24 hours per day in full autonomous mode for 1 week on two separate occasions, the instrument was able to detect relevant target concentrations of up to 100,000 particles per liter of air within the size range of interest. Detection time was on the order of 1 minute with a greater than 90 percent probability of detection. There was one false-positive during the 14 days of testing, and the instrument failed one time when the inlet clogged as a result of the extremely high particle concentration used in the test. From these data, Gard concluded that this technology is at TRL 5, perhaps approaching TRL 6.

Describing the weaknesses of SPAMS, Gard said that the harshness of the ionization method reduces the information content of the fingerprint, restricting it largely to metabolic signals. The system does not have predictive capability for signatures in this mass range; therefore, the system needs to be trained for each signature, given the lack of understanding of how genomic and proteomic information translates into metabolic products. Finally, the cost and size of this system are too great for widespread deployment.

The strengths of the system are that it has a broad range of potential signatures, given the generality of the ionization technique, and that analysis times are in the microsecond range with detection times of less than a minute. Contextual information provided by the system in terms of

particle size, concentration, temporal profiles, spatial distributions, and the potential to see other additives can indicate whether a release was intentional. In addition, SPAMS can detect other threats, such as chemical and radiological releases.

With its low false-alarm rate and the ability to generate data rapidly, SPAMS could be used to initiate modeling and low-level data-gathering activities in preparation for a potential BAR event, Gard said. In the near term, he argued in closing, the atmospheric context that this technology produces and the speed with which it produces that information can support decision making and response activities that support data from high-confidence detectors. By preserving the aerosol characteristics of the sample, this technology allows one to "translate" between the biology and its implications in terms of transport and potential consequences. This information would fill what Gard said is an important gap in the BioWatch system. "I think this intermediate detection specificity has not really been looked at in terms of how it could be useful within the network and supporting the decision makers," he said.

Miniature Mass Spectrometry System for Microorganisms

Mass spectrometers do not have to be large, said Zheng Ouyang, associate professor at Purdue University, but mass spectrometers cannot access samples directly and therefore need to be interfaced with sample preparation purification and injection modules that add to the size and complexity of the total system. For example, an instrument that he and his colleagues developed is about the size of a large box of tissues and weighs about 4 kilograms (Gao et al., 2008). However, this device functions at atmospheric pressure, and so any biomarker larger than a fatty acid molecule would not be volatile enough to enter the mass spectrometer for analysis. He said that he does not believe it is possible to miniaturize a universal mass spectrometry platform, but it is possible to miniaturize a system for a specific application. As an example, he described a system that his group developed for monitoring therapeutic drug levels from a drop of blood in about 60 seconds.

How might this approach be applied to the analysis of microorganisms in aerosol droplets? Zheng proposed starting with a disposable furnace filter rated for microorganisms fitted inside a small cartridge. Air would be drawn through the filter, which would collect particles. After a suitable collection time, the cartridge would be inserted into the machine for paper spray mass spectrometry analysis (Yang et al., 2012).

Before discussing what might be observed using such an approach, Zheng said that there are more than 40 research systems available today that can do ambient ionization—that is, ionization without any sample preparation—up from 2 that were available in 2005. More than 10 companies have produced miniature mass spectrometers, but most of these can only handle gas samples. The key questions, he said, are whether any of these systems can identify biomarkers that can be efficiently sampled and ionized by ambient ionization methods and that can be used for high-specificity characterization and identification of bacteria.

He believes that lipid profiles may be the answer, and he presented data experiments demonstrating real-time identification of bacteria growing in a biofilm using both positive and negative ion modes to create lipid fingerprints (Song et al., 2009). These experiments demonstrated that it is feasible to differentiate subspecies, too. More recent work has shown that the same lipid fingerprint is obtained from organisms collected on filter paper and analyzed using paper spray mass spectrometry.

According to Zheng, none of these methods is ready for Tier 1, but he believes that with further work, lipid-based identification using ambient ionization on small mass spectrometers could be Tier 2 or Tier 3. What is needed to start this development effort, he said, is the provision of standard samples of microorganisms to generate a large body of spectra in order to optimize statistical data analysis methods. From there, it would be necessary to optimize sampling ionization conditions, characterize matrix effects and detection limits, and determine if the method can identify multiple microorganisms in the same sample.

Discussion

Raymond Mariella, Jr., asked if either fatty acid esters or the low mass-to-charge ratios measurable with relatively simple instruments have the ability to generate fingerprints that can meet the BioWatch criteria of detecting a thousand organisms of interest against a background of 1 to 10 million other organisms with a false-positive rate of 10^{-7} or better. Gard replied that for the system he has been working with, the answer is no, and that is one reason that he sees that particular method as an orthogonal technology that would provide atmospheric context and other information that would benefit BioWatch. Gard also said that he has not seen any mass spectrometry system that can meet those specifications. Mariella agreed that these systems would have value for orthogonal de-

tection, but he was curious if they could serve as primary detection systems as well.

Rabih Jabbour said that proteomic mass spectrometry is more likely to reach the needed level of specificity, and Johnson added that what he likes about whole-organism protein mass spectrometry is that this field is moving forward without requiring government investment. He predicted that these instruments, which now cost about $200,000 and a $2 cost per sample, will be miniaturized to a point-of-care level. "If you want to move into environmental detection 4 or 5 years from now, you would probably do that much more easily than trying to design a system for this purpose now," he said.

Cindy Bruckner-Lea pointed out that SPAMS could be valuable for monitoring indoor releases because it provides the possibility of real-time detection, which could be used to trigger follow-on analysis. She indicated that a cost-benefit analysis would be needed, however, and a major limitation is the very high cost of these systems. She questioned whether SPAMS would be able to have enough sensitivity for outdoor settings, because the limit-of-detection requirements are more challenging for outdoor compared with indoor releases (unless there is a very high density of detectors outdoors).

Thomas Companion raised the point that mass spectrometry is one of the primary technologies used to detect trace explosives, which are of very high molecular weight and with virtually no vapor pressure, so there are some similarities between that and detecting large biomolecules. What DHS found was that how sampling was done was more important than the device that was used to analyze the sample. He suggested that investments should be made in the area of sampling. Snyder said that, given the complexity of environmental samples compared with clinical samples, he supported that idea.

5

Final Thoughts

In the workshop's final session, the session rapporteurs presented their reflections on the workshop. Michael Walter, Detection Branch chief and BioWatch program manager in the Office of Health Affairs at the Department of Homeland Security, also provided his concluding remarks. This chapter summarizes these comments and the ensuing general discussion among the workshop participants; Table 5-1 provides an overview of the four families of technology.[1]

THE PUBLIC HEALTH PERSPECTIVE

Beth Maldin Morgenthau, assistant commissioner for the Bureau of Policy, Community Resilience and Response within the Office of Emergency Preparedness and Response, New York City Department of Health and Mental Hygiene, said that the key message that came from the public health session was that when faced with a BioWatch Actionable Result (BAR), decision makers want more information as quickly as possible to help them put that BAR into context. Although each jurisdiction may have its own process for responding to a BAR and reaching a decision on what steps to take next, all of those processes benefit from having as much information as possible, whether it be from human and animal

[1]The topics highlighted in this chapter are based on the summary of remarks discussed during each session. Additional comments by participants related to the closing remarks are also included. As noted in Chapter 1, comments included here should not be construed as reflecting any group consensus or endorsements by the Institute of Medicine or the National Research Council.

TABLE 5-1 Potential Families of Technology for an Autonomous Detection System for BioWatch

	Nucleic Acid Signatures	Immunoassays and Protein Signatures	Genomic Sequencing	Mass Spectrometry
TRL of integrated systems using this technology	**TRL 9**	**TRL 6-plus**[a]	**TRL 4**[b]	**TRL 6**
Sample preparation	**Required** • Cell lysis, nucleic acid extraction	**Minimal** • Cell lysis increases sensitivity	**Required** • Cell lysis, nucleic acid extraction	**No**[c] • Less sample may improve sensitivity and specificity
Detectable agents	**Nucleic acids of targeted agents** • Bacteria, viruses, fungi, indirect detection of toxins • Limited to included probes	**Protein and structural epitopes, nucleic acids, toxins of targeted agents** • Bacteria, viruses, fungi, direct detection of toxins • Limited to included probes	**Nucleic acids, known and novel** • Bacteria, viruses, fungi, indirect detection of toxins • Limited by bioinformatic algorithms/ databases	**Chemical signatures** • Bacteria, viruses, fungi, toxins, chemical agents • Limited by bioinformatic algorithms/ databases
Sensitivity (cfu/m^3)	**High** • Single-copy detection for some organisms	**Moderate** • Some exceed current BioWatch requirements	**High** • Enrichment strategies are needed for low-titer samples	**High** • May depend on sample complexity
Specificity (false positive rate/ speciation)	**High** • False positive rate of 10^{-7} or better	**Moderate** • Near-neighbor discrimination	**High** • Capable of single-nucleotide discrimination	**Moderate** • Not as high as nucleic acid signatures

Processing time for detection	**<10 min (cycle times)** • 4–6 hrs (collection, prep, and cycle times)	**Typically 3-10 minutes** • Range: <5 minutes–60 minutes	**>1 hour** • >10 hours (collection, prep, cycle times, analysis)	**Near real time** • Approx. 1 minute
Relative operational cost	**Moderate** • Consumables and bioinformatic costs	**Inexpensive** • <1 cent for each analysis • <$100,000–$150,000 per unit	**Expensive** • Consumables and bioinformatic costs	**Inexpensive** • Few consumables
Operational environment	**Indoor/Outdoor**	**Indoor/Outdoor** • Less sensitive to dirty settings than nucleic acid–based technology	**Currently in clinical and research laboratories**	**Indoor/Outdoor**
Ability to test independently	**Yes** • Sensitive enough for trigger, specific enough for confirmation	**No** • Used as trigger, with PCR confirmation	**No** • Used for ultimate confirmation, identification	**No** • Used as trigger with PCR confirmation

[a] These tests are commercially available and FDA-approved in some configurations, such as lateral flow assays. The "-plus" is intended to reflect this.

[b] The lack of availability of an integrated system for sequencing is not simply due to technology limitations. No one has tried to engineer such a system due in large part to the newness of component subsystems.

[c] Although smaller, less complex samples improve sensitivity and specificity, tiny samples reduce sensitivity, due to the effects of Poisson sampling errors for low-concentration agents. Specificity is reduced once the virus, bacteria, or fungus is fragmented by the ionization process.

NOTE: cfu/m^3 = colony-forming units per cubic meter of air; FDA= Food and Drug Administration; PCR = polymerase chain reaction; TRL = technology readiness level.

surveillance, weather, past history, law enforcement information, or the results of other laboratory or field tests. The panelists from that session also noted that, in the end, their decision-making process is an art. "We will be making critical decisions without understanding the viability of the agent, the sensitivity to countermeasures, if this is an isolated incident or part of a series of incidents, when and where the release took place, or how much has this been modified or weaponized," Maldin said. "We are making these decisions without really understanding what the impact is to public health and just making the best guess we can."

This decision-making process becomes more complicated still when a BAR comes from an indoor BioWatch detector, which would trigger environmental sampling and require closing a facility. This is a potentially high-regret decision that instantly makes the BAR highly public at a time when an investigation is just beginning. If BioWatch plans to focus on indoor detection, it must do so in close cooperation not only with local public decision makers, but also with facility and transit officials, Maldin said.

Along the same lines, Maldin noted that the panel stressed the importance of public health laboratorians and decision makers being comfortable with whatever detection system BioWatch ultimately chooses. "We heard from the panel that there is no room for error. Public health's currency is our credibility. We can't make a mistake, and so we need to have complete confidence in the system." Whatever system BioWatch chooses, she said, it must be the same or better than the current system in terms of sensitivity, specificity, and reproducibility.

Desired Features of Future BioWatch Technology

The main items on public health's wish list for BioWatch were a shortened turnaround time to aid the decision making that must happen in the immediate aftermath of a BAR notification; higher spatial resolution in terms of where an agent was released and at how many sites; characterization that would indicate whether an agent is pathogenic or not, viable or not, weaponized or not, and susceptible to medical countermeasures or not; and an estimate of how much agent was released. Also on the wish list were the ability to access an instrument remotely to obtain instrument-specific performance data that would help put a BAR in context, as well as the ability of the BioWatch detectors to store a sample for additional testing and criminal investigation. Any future BioWatch

system should undergo extensive quality-assurance testing with the involvement of public health laboratorians, Maldin reiterated.

Flexibility in being able to add or remove agents based on intelligence and scientific information is important, but so too is guidance on how public health can respond to any new agent, she said. "Don't test unless you know what you are going to do with the results," was an important message noted by the panelists, Maldin said, as was public health's need for the resources to respond to a threat. "If the alarm goes off and there is no one there to respond, then the system is worthless," she said in commenting on the funding issues that most, if not all, public health departments have endured over the past number of years. For example, since 2005 Chicago has cut its public health staff from 1,600 to 600, while Texas has cut its staff from 700 to 500. Because the next-generation BioWatch system is going to be justifiably more complex and sophisticated, it will place an even greater burden on public health. "Not even the current resources will be able to support that," Maldin said. "We need to think about how we are going to rebuild public health capacity."

In closing, Maldin said that the bottom line is that public health is in the business of saving lives and that it finds itself in the position of making critical decisions with little information. "If we get it wrong and overreact, the economic and political impacts of unnecessarily shutting down a facility or transportation are huge," she said. "If we get it wrong and we underreact, the public health impact of doing nothing for those that have been exposed or delaying that are even bigger." Public health is dedicated to getting the next-generation BioWatch system right and wants to be help design that system so that it provides the information that the end user—the public health decision maker—needs.

NEXT-GENERATION DETECTION TECHNOLOGIES

Nucleic-Acid Signatures

William O'Neill, development program manager and project engineer, Biohazard Detection System, USPS, said that there are three systems—the microfluidic bioagent autonomous networked detector (M-BAND), the autonomous pathogen detection system (APDS), and the biohazard detection system (BDS)—that have provided effective aerosol collection, have demonstrated complex and automated sample preparation, have performed multiplexed nucleic acid analysis, are TRL 8 or

better, and have been in the field and running. All of these systems include detailed reporting, automated chain-of-custody archiving, secure network communications, extensive diagnostic reporting, and detailed logistic support strategies. They also have various challenges in terms of incorporating new assays, given the expectations of zero false-positives from environmental samples, which will require extensive field testing prior to implementation, and there could be challenges regarding supply-chain issues if these systems are deployed extensively.

The major challenge, according to the panel presentations and comments, will be the integration of multiple technologies from various vendors. In O'Neill's view, it will be necessary to perform detailed systems engineering involving the technology developers to maximize system sensitivity in a cost-effective package. Two important technology challenges, he said, are maximizing sample concentration and minimizing sample contamination in a system with high reliability. In order to meet this last requirement, he said, extensive and repeated field testing with detailed performance analysis will be a necessity. This can only occur as a sustained, multiyear effort, accompanied by a funding commitment from the BioWatch program, an idea that Eric Eisenstadt, independent consultant, noted as well.

Although there are some issues with several of its assays, the real-time multiplexed polymerase chain reaction (PCR) has compiled an extensive track record of accurate reporting. Thomas Slezak, assistant program leader, Informatics for the Global Security Program, Lawrence Livermore National Laboratory (LLNL), emphasized that even though BioWatch's PCR-based system may have misidentified *Francisella tularensis* in field tests, that was a failure of the specific assay, not of the overall design of the BioWatch detection technology. The panelists agreed that PCR-based testing can serve as a primary detection assay and that false-positives can and must be minimized with orthogonal testing by any one of the many other analytical systems described by the panel. Besides their proven track record, PCR assays have well-established supply chains that can provide large quantities of the necessary reagents. One issue that must be addressed is sample preparation, particularly with regard to the ability to detect the species in differing environments, O'Neill said. Fluidics technologies may offer a solution to this problem. Another issue with PCR is the cost related to the licensing burden that comes with this technology. He noted, however, that the majority of the relevant patents will expire by 2016.

Immunoassays and Protein Signatures

For immunoassay and protein technologies, the NG-ADS (next-generation autonomous detection system) array system is currently at TRL 6-plus and is capable of running immunoassays for rapid detection and nucleic acids for confirmation. What Thomas Slezak thought was important about NG-ADS is that this is largely an off-the-shelf system with an extensive product development history behind it. There was general agreement among the panelists that antibody-based detection is potentially fast but that it is not likely to achieve the sensitivity and specificity of nucleic acid–based technologies in a stand-alone next-generation BioWatch system, or at least not before 2020. However, because of their cost, speed, ability to be automated and operate in a wide range of environments, and well-developed technology base, such systems could find a role in combination with other technologies. Slezak added that given that these technologies all depend on recognizing surface epitopes, they will not be as good as genomic techniques at characterizing virulence and antibiotic resistance, which is another reason why antibody-based technologies are not likely to be used for primary detection. However, protein-based assays should be better than nucleic acid technologies at detecting and characterizing toxins.

Slezak stressed the importance of assay validation and cautioned that it should never be taken lightly. He added that the community should consider ideas for better validating highly multiplexed assays, and he said that protein-based technologies are undergoing field validation and are performing well, although much more extensive testing still lies ahead. He was particularly impressed by the ability of Raman-based systems to detect both biological and chemical threats in the atmosphere, and he thought that the single-molecule array-based detection system had potential as well. One additional message that he heard from the panelists was the need for the Department of Homeland Security (DHS) to look beyond the familiar technologies and familiar development pathways.

Genomic Sequencing

One reason it is important to continue technology development even though PCR technology has proven to be a workhorse technology, O'Neill said, is the need to address the anticipated genetically engineered threats. O'Neill and Eisenstadt both said they were impressed with the new genomic technologies, particularly metagenomics and pan-genomics;

these technologies can potentially address this issue with automated processing systems as previously discussed. Both O'Neill and Eisenstadt also said that they were encouraged by the significant effort being conducted to sequence large numbers of microorganisms and that they were intrigued by the idea of using nanopores to provide direct, reagent-free sequencing of a genome. O'Neill voiced the concern that nucleic acid technologies could produce so much information that it would add many levels of complexity that public health decision makers would then have to absorb. He expressed the opinion that the biodefense community needs to devote time and energy to develop guidelines that can help public health deal with this information. Eisenstadt agreed, reiterating how important it will be to involve laboratorians and public health decision makers early in the development process of these new nucleic-acid technologies. He then noted that the real advantage of genomic sequencing technologies is that they provide the most complete and accurate view of the multiple organisms in the environment and the dynamic sequence space that they occupy. "The digital representation of the organism that you get via genome sequencing provides you with material that you can query to answer many questions, ranging from forensics to therapies to treatment to developing vaccines," Eisenstadt said.

Improvements in genomic sequencing, as is the case with the other characterization technologies that were discussed, will be driven by clinical applications, particularly for the point-of-care setting, Eisenstadt said. While the clinical opportunities are attracting funding that this biodefense community can leverage, they are also raising technology awareness within the public health community. Genomic sequencing will not, however, be a panacea for biodetection, Eisenstadt said. "What is the proper role for genome sequencing in real-time or near real-time detection when you are trying to monitor or identify when someone has deliberately introduced a pathogen into your outdoor or indoor environment? We didn't resolve that here." It is still unclear, he added, whether genomic sequencing will be deployed in the field or will remain a laboratory-based system for confirmation and further elaboration of signals from primary detectors. Cost and training are potential challenges for genomic technologies, and the field is evolving so quickly as to create a challenge for any acquisition programs such as BioWatch, particularly when it comes to validating the technology in the face of rapid change.

Eisenstadt reiterated the need to develop automated and reliable sample preparation technologies. He also highlighted the need to construct and maintain high-quality, curated databases and to develop the

bioinformatics tools that will be needed to make sense of the large datasets that most of these new technologies, genomic and others, will generate. As O'Neill discussed, integration is a challenge that needs to be addressed. Eisenstadt highlighted the development of aerosol-to-sample analysis technologies and the aerosol characterization processes as having great potential. He expressed concern that the BioWatch program needs to ensure that there is enough flexibility in the acquisition plan that it will not miss out on capitalizing on the genomics wave when it peaks.

Mass Spectrometry

According to Charles Kolb, president and chief executive officer, Aerodyne Research, Inc., a strength of mass spectrometry is its ability to characterize both targets and the background—an important capability because the issue BioWatch faces is not signal-to-noise but signal-to-background. One way to address that challenge is to concentrate the target as much as possible and to reduce background content, while another is to understand the background as much as possible, and it is at this second approach that mass spectrometry excels. Another strength of mass spectrometry is that it is supported by a huge technical base of instrument development. After reviewing the basic mass spectrometer components, Kolb said that the available technology affords a wide range of choices in terms of cost, size, maximum resolution, and other characteristics. He offered the opinion that while real-world mass spectrometry applications are evolving rapidly, it will take a number of years to settle on the best combinations of technology and data analytics for BioWatch and then field test the resulting instruments.

Nonetheless, Kolb said, the panelists pointed out several areas in which mass spectrometry could play a role in BioWatch. As a real-time sentinel, mass spectrometry could provide a signal that would trigger more specific technologies as it detects suspicious particles. It could also provide background characteristics that could be used to correlate problem particle types with wind direction, temperature, and diurnal and seasonal variations and to provide real-time data to allow back-dispersion modeling to characterize release and forward-dispersion modeling to predict impact areas. With further technology and database development, mass spectrometry may be able to improve detection specificity.

THE LABORATORY PERSPECTIVE

As a potential end user of the technology that had been discussed during the course of the workshop, Sandra Smole, director of the Division of Molecular Diagnostics and Virology in the Massachusetts Department of Public Health's Bureau of Laboratory Science, said that she was excited about these technologies but she was "very concerned that many of them will not or do not meet the state of readiness that is required for the BioWatch autonomous detector and, certainly, not the state of readiness required for deployment in the BioWatch system." From an operational perspective, she said, the public health response actions that are initiated as a result of these technologies must be supported by a robust system meeting all of the technical specifications that are listed in the DHS request for proposals as well as the needs of the public health laboratorians and decision makers. As a laboratorian she is most interested in understanding the performance characteristics of these systems and then being able to network into and access these systems to monitor their performance. The most critical information needed from an autonomous system is the confident identification of the biological threat to inform follow-on actions and laboratory analyses.

One of the most important roles of the laboratorian is to explain the results that are delivered to the public health decision makers. From that perspective, having multiple detection capabilities that can provide information on drug resistance markers, viability, and virulence can be very useful. "As a laboratorian, I am very interested in strain typing and fingerprinting," she said. "Public health laboratorians spend a considerable amount of effort in tracking down the source of infection and outbreaks, and I am very interested in applying technologies that allow for that capacity." In particular, she noted the potential of mass spectrometry and genome sequencing to provide that type of information, but she also noted that developing and networking well-curated databases will be critical for realizing that potential. In addition, she commented that this detailed characterization would not be needed immediately as part of an autonomous system, but could be provided by the lab-based investigation. She also reiterated the comment made by several speakers that BioWatch can benefit from the efforts of the clinical diagnostics community and the efforts that are going into developing products for those end users.

As a final comment, Smole agreed with the general discussions that nucleic acid–based technologies are currently at the high technology

readiness levels, but she made a plea for the developers of other technologies to continue their work in this field. "See if you can try to develop these technologies and bring them up to speed and compete," she said, while acknowledging that such an effort will require funding that may be hard to procure.

DISCUSSION

Toby Merlin from the Centers for Disease Control and Prevention (CDC) started the discussion by asking the workshop attendees to think about the role that technology can play in managing risk, that is, by providing information that enables the decision maker to make the best possible decision in the face of uncertainty. In that regard, he noted the importance of high-quality, highly trusted data for the decision maker. Increasing the confidence that the decision maker has in the quality of the data that the technology provides lowers the decision maker's risk. He also pointed out to the audience that while this workshop focused on aerosol release, there are many other avenues of release that BioWatch does not cover.

Suzet McKinney, deputy commissioner for the Chicago Department of Public Health, said that she has been involved with BioWatch since 2005 and that this was the first time that she could remember public health having the opportunity to have a discussion with both DHS and industry at the same time. "Public health would like to be involved and would like to continue to be involved in this discussion," she said. Speaking to the technology developers, she said that while the technology development community wants to test these technologies in an operational environment, she and her public health colleagues do not want to see the results of those tests in real time. "If we see them during the test, we have to act on them," she said. "Until we have the confidence in these technologies that the actions are sound based on the fact that the technology works, we are not going to want to go there." Scott Hughes from the New York City Public Health Laboratory added that it is important to share the data from these tests eventually so that he and his colleagues can become familiar with the data and gain confidence in them.

David Persse, emergency medical system director for the City of Houston, said that one piece of information that public health needs to makes its decision is how different a result is from the local environmental background. He reminded the audience that when BioWatch was

launched and had its first BAR, there was not a clear understanding of whether that result was due to the presence of microorganisms that naturally existed in the environment or pathogen purposely introduced into the environment. Persse said that context is an important factor for public health decision makers to consider; for him, this information is important when explaining decisions to his mayor. Erica Pan, director of the Division of Communicable Disease Control and Prevention in the Alameda County (California) Public Health Department, added that it is not just the mayor who needs to understand the reasons behind public health's decision; the public does, too.

Jeffrey Schloss from the National Human Genome Research Institute asked how feasible it would be to swap out modules in a deployed instrument because it is likely that inserting a new detection technology would also require inserting a new sample-processing module. Another participant asked if there was some metric for how big an improvement would need to be to result in swapping out technology, given the time and cost involved in making such a transition. O'Neill answered the first question by saying that such flexibility is possible but that any transition would take 12 to 18 months, given the thousands of units that would have to be tested and validated and the training that would have to take place. Slezak agreed with that assessment. "Switching out a nucleic acid PCR-based system for any of the other wonderful technologies we heard here is ground zero," he said. "You would have to build a whole new system from scratch and test the whole system in parallel with the existing one." Allen Northrup, chief executive officer of the Northrup Consulting Group, disagreed with this assessment, commenting that one way to improve modularity is to understand the basic operating parameters of the existing system in terms of inputs and outputs and to design replacement modules to fit those parameters from the beginning.

William Raub, chair of the workshop planning committee, asked if the idea of combining two orthogonal testing methods in the same instrument was too big a challenge in terms of integration and operating reliability. He wondered if a better approach might be to design a semi-autonomous system that would require a laboratorian to make the decision whether to trigger a second detection method based on the results of the primary method. John Vitko, who moderated the first technology session on nucleic acid signatures, said he was skeptical about the practicality of integrating two detection systems on the same deployable platform in part because of cost, but largely because of the difficulty of optimizing two completely different technologies to run in the same instrument.

THE BIOWATCH PERSPECTIVE

This workshop, Michael Walter said, was incredibly important for the BioWatch program because it provided the opportunity for the biodetection field to examine the whole range of technologies, from mature to developing, and to get a sense of the technology pipeline so that BioWatch program officials can make an informed decision on those areas that it should focus on. He also reminded the workshop participants of the complexity of the BioWatch program. "It has an enormous number of moving parts, and the ramifications of the decisions it makes are enormously important," he said. "When we look at how we are going to move forward with the BioWatch program, it is important to remember that it has got to fit into the public health realm, and to do that it has to be better, faster, cheaper. Those are our three mantras that Congress assigned to the program. That is what we need to look at."

At the same time, the BioWatch program cannot fund research and development—it is concerned solely with deploying an operational system that generates accurate, actionable results for use by the local public health jurisdictions. He noted that there was some concern voiced at the workshop about where information from the BioWatch detectors would go, and he stressed that this is a nonissue, as all information goes to the public health departments in the local jurisdictions. It may happen, he said, that CDC would get the information simultaneously, but he stated clearly that "the information will remain under the control of the state and local public health departments."

In commenting on the prime requirements for technology for BioWatch, Walter said that "the last thing we can do is test, get a result, test, get a result, and test again. We don't have the time for it and we don't have the money for it. Whatever we put out there has got to be fast, accurate, and has got to be right." He went on to say that the program is not allowed a false alarm, for that could jeopardize the program, and he acknowledged the extreme scrutiny that the program is coming under from Congress, the White House, and DHS. He also remarked that the procurement philosophy for BioWatch holds that while it may deploy a particular technology in an autonomous system, it will replace modules within that system as the technology improves and is validated in the field. As a result, BioWatch will continue to be interested in technology innovations in the years ahead.

A

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B

Biographical Sketches of Workshop Participants

William F. Raub, Ph.D. (*Chair*), retired in January 2009 after more than 42 years in the employ of the federal government, primarily the Department of Health and Human Services (HHS). Current activities include advising the U.S. Postal Service on public health emergency preparedness, advising HHS on vaccine safety infrastructure, serving as adjunct staff for the RAND Corporation, serving on the science advisory board of George Mason University, and performing volunteer work for St. John's Church, Chevy Chase, Maryland. Dr. Raub held a wide variety of positions within the federal government, including science advisor to the secretary of HHS (1995–2009); science advisor to the administrator of the Environmental Protection Agency (1992–1995); special assistant within the Office of Science and Technology Policy, Executive Office of the President of the United States (1991–1992); acting director, National Institutes of Health (NIH) (1989–1991); and deputy director, NIH (1986–1991). Dr. Raub received numerous awards, including the Presidential Distinguished Executive Rank Award, the Presidential Meritorious Executive Rank Award, the HHS Distinguished Service Award, the American Medical Association's Nathan Davis Award, and the Society of Research Administrators' Award for Distinguished Contribution to Research Administration.

Amy Altman, Ph.D., joined Luminex Corporation in March 2007 and is currently the vice president of biodefense and food safety. As vice president, she is responsible for building and managing the Luminex biodefense business segment. This involves programs that encompass environmental monitoring for biological threat agents, both autonomous and lab based, as well as applications in the medical diagnostic area, such as

for first responders and for syndromic surveillance applications. Prior to being appointed vice president, Dr. Altman served as director of the Extramural Research Office and most recently as senior director of research and development for the Luminex Bioscience Group, where she was responsible for directing the development of multiplexed assays on proprietary xMAP technology for both the research and clinical diagnostic market. Prior to joining Luminex, Dr. Altman was an associate with Booz Allen Hamilton, a global strategy and technology consulting firm. As an associate with Booz Allen, Dr. Altman provided science and technology support to clients within the Department of Defense (DoD), generally focused in the areas of bioterrorism (threat assessment, sensing, defeat, and decontamination of weapons of mass destruction), enhancing soldier performance and survivability, and applications of biotechnology to DoD. She also served as a subject-matter expert in the area of chemical, biological, radiological, nuclear, and explosives. Dr. Altman received her B.A. and M.S. degrees in microbiology from Miami University in Oxford, Ohio. She received her Ph.D. in molecular biology from Vanderbilt University in 2000 and completed a 3-year postdoctoral fellowship in the area of eukaryotic DNA replication and cancer biology. Dr. Altman also received a certificate in business for growing and managing the biotech enterprise from the Vanderbilt Owen Graduate School of Management in 2003.

Andrew P. Bartko, Ph.D., received a B.S. from the University of Pittsburgh in 1997 and a Ph.D. in physical chemistry in 2002. His graduate work consisted of deciphering spatially heterogeneous relaxation dynamics of glass-forming systems using novel rotational single-molecule microscopy techniques. In 2002 Dr. Bartko joined the soft-matter nanotechnology and advanced spectroscopy team at Los Alamos National Laboratory, where he studied the ultrafast photophysics of semiconducting quantum dots. Dr. Bartko is a senior scientist in Battelle's Technology Development Group, where he contributes to several applied spectroscopy efforts that focus on biological and chemical sensing. Dr. Bartko is the manager and technical leader of an interdisciplinary team that is developing Battelle's Resource Effective Bio-Identification System (REBS). The rapid microbial sensing capabilities of REBS have been shown to have practical and strategic importance where rapid, accurate, and precise microbial contamination control is required. Dr. Bartko has developed several rapid microbial control applications for the defense, security, and industrial markets.

Thomas A. Cebula, Ph.D., earned his B.S. in chemistry from Wilkes College (Wilkes-Barre, Pennsylvania) and his Ph.D. in biology (biochemical genetics) from Johns Hopkins University (Baltimore, Maryland). After postdoctoral work in cellular microbiology at the McCollum-Pratt Institute and Johns Hopkins University School of Medicine, Dr. Cebula was recruited to initiate a research program at the Food and Drug Administration (FDA). Dr. Cebula built a successful program, serving as chief of the Molecular Biology Branch, director of the Division of Molecular Biology, and director for the Office of Applied Research and Safety Assessment. His research and leadership skills were recognized with 35 U.S. government awards, including FDA's Commendable Service Award, Award of Merit, and Outstanding Science Achievement Award, and the Department of Health and Human Services Distinguished Service Award. Dr. Cebula is a former member of the U.S. Senior Biomedical Research Service and the Senior Executive Service. His laboratory was designated a Department of Homeland Security Center of Excellence for Microbial Forensics of Enteric Pathogens. Dr. Cebula is a visiting professor of biology at Johns Hopkins University, an adjunct professor of microbiology at the University of Maryland, and the chief technical officer at CosmosID®, Inc., a bioinformatics and genomic data mining company. He is a member of Johns Hopkins' Society of Scholars and a fellow of the American Academy of Microbiology. He serves on the advisory board of the Pathosystems Resource Integration Center at the National Institutes of Health and served previously on National Research Council and Federal Bureau of Investigation committees that addressed conduct and funding of counter-bioterrorism research. He is a former chair of the White House Office of Science and Technology Policy Working Group on Bacterial Genomics and member of the Office of the President's National Science and Technology Council's Microbial Forensic Task Force. Dr. Cebula has authored more than 100 peer-reviewed articles and a like number of published abstracts.

Rita Colwell, Ph.D., is chairman of Canon U.S. Life Sciences, Inc., distinguished university professor both at the University of Maryland, College Park, and Johns Hopkins University Bloomberg School of Public Health, and president and founder of CosmosID, Inc. Her interests are focused on global infectious diseases, water, and health, and she is currently developing an international network to address emerging infectious diseases and water issues, including safe drinking water for both the developed and developing world. Dr. Colwell served as the 11th director

of the National Science Foundation (NSF) from 1998 to 2004. In her capacity as NSF director, she served as cochair of the Committee on Science of the National Science and Technology Council. Her major interests include K–12 science and mathematics education, graduate science and engineering education, and the increased participation of women and minorities in science and engineering. Dr. Colwell has held many advisory positions in the U.S. government, nonprofit science policy organizations, and private foundations as well as in the international scientific research community. She is a nationally respected scientist and educator and has authored or coauthored 16 books and more than 700 scientific publications. She produced the award-winning film *Invisible Seas* and has served on editorial boards of numerous scientific journals. Before joining NSF, Dr. Colwell was president of the University of Maryland Biotechnology Institute and professor of microbiology and biotechnology at the University of Maryland. She was also a member of the National Science Board from 1984 to 1990. Dr. Colwell has previously served as chairman of the Board of Governors of the American Academy of Microbiology and also as president of the American Association for the Advancement of Science, the Washington Academy of Sciences, the American Society for Microbiology, the Sigma Xi National Science Honorary Society, and the International Union of Microbiological Societies. Dr. Colwell is a member of the National Academy of Sciences, the Royal Swedish Academy of Sciences, Stockholm, the American Academy of Arts and Sciences, and the American Philosophical Society.

Chris Detter, Ph.D., received his Ph.D. in molecular genetics and microbiology from the University of Florida and has 18 years of relevant hands-on experience in the field of high-throughput sequencing and analysis. Dr. Detter has supported the scientific and program development as well as implementation of new capabilities and strategic directions for biothreat and biodefense mission areas. This includes, but is not limited to, areas such as biosurveillance, data to decision, and high-throughput genomics (sequencing, metagenomics, and genome analysis technologies). He has authored or coauthored more than 100 peer-reviewed publications in the field of high-throughput genomics and DNA sequencing. Dr. Detter and his teams have shotgun sequenced more than 2,000 prokaryotic, eukaryotic, and viral genomes; have made subclone libraries from more than 10,000 BioWatch Advisory Committees as part of the Human Genome Project; have finished the sequencing of more than 800 microbial genomes for the Department of Energy (DOE), De-

partment of Defense (DoD), and Department of Homeland Security (DHS); and have helped introduce next-generation sequencing and analysis capabilities to international government labs in Georgia, Jordan, Kenya, and Thailand. Dr. Detter has led genomics and technology development groups while stationed at the DOE–Joint Genome Institute in Walnut Creek, California, and Los Alamos National Laboratory (LANL) in Los Alamos, New Mexico. Dr. Detter is currently the biothreat/biodefense program director for bio-related DoD, DHS, and intelligence programs at LANL.

Eric Eisenstadt, Ph.D., has been an independent technical consultant since March 2010. He provides scientific and technical advice to the Air Force Research Laboratory, the Defense Advanced Projects Research Agency (DARPA), the Defense Threat Reduction Agency, and the Department of Homeland Security. Prior to becoming a consultant, Dr. Eisenstadt was the vice president for research at the J. Craig Venter Institute and the Institute for Genomic Research (2005–2010). He also served as a program manager at DARPA (1999–2005), where he developed and managed basic and applied interdisciplinary research programs in various biotechnology areas, such as genomic sequencing of pathogens, neurobiology, synthetic biology, and protein design. Before joining DARPA, Dr. Eisenstadt was a program officer at the Office of Naval Research (ONR) from 1988 to 1999, where he developed and managed basic research programs in marine biotechnology (including a focus on life at high temperature and pressure), systems biology (with a focus on developing novel computational approaches to modeling biological systems), anaerobic bioremediation processes, and biomineralization. While at ONR, Dr. Eisenstadt was the Navy representative to the Joint Service Technical Panel for Chemical and Biological Warfare Defense and served on ONR's Historically Black Colleges Committee. Before his government service, Dr. Eisenstadt was a member of the faculty at the Harvard University School of Public Health in the Department of Microbiology and the Laboratory of Toxicology, where he taught and investigated mechanisms of mutagenesis and DNA repair in bacteria and yeast. Dr. Eisenstadt received his A.B. and Ph.D. in biology from Washington University, St. Louis, and did postdoctoral work at the Université de Paris, Orsay (as an National Science Foundation/North Atlantic Treaty Organization postdoctoral fellow), at the Universität zu Köln as a Deutsche Forschungsgemeinschaft postdoctoral fellow, and at the Labor-

atory of Molecular Biology, National Institute of Neurological Diseases and Stroke, as a National Institutes of Health staff fellow.

Eric Gard, Ph.D., leads the Lawrence Livermore National Laboratory's Defense Biology Division. His areas of expertise include mass spectrometry, aerosol science, atmospheric chemistry and transport, biology, and chemistry. Dr. Gard has designed, built, and field-tested a series of innovative mass spectrometers for the chemical and biological analysis of proteins, saccharides, and many other complex biological molecules. The equipment has ranged from dedicated lab-based equipment for more traditional biological analysis applications to transportable systems for real-time aerosol analysis. Dr. Gard's most recent work includes building systems for real-time detection and identification of aerosolized biological and chemical agent–containing particles using mass spectrometry. These efforts have spanned the past 25 years and have included leading projects for the Department of Energy, the Defense Advanced Research Projects Agency, and other Department of Defense organizations. Dr. Gard received his Ph.D. from the University of California, Davis.

David Hanlon, Ph.D., joined Quanterix in 2008 to identify unmet market opportunities that would benefit from the company's technology platform. Prior to joining Quanterix, Dr. Hanlon led several external research collaborations at Cytyc to discover novel biomarkers of cervical neoplasia as well as an internal team focused on marker validation and the development of diagnostic assays. Dr. Hanlon was the director of research and development at VisEn Medical and project manager at Proteome, where he led a team of scientists developing protein-centric databases for comparative and functional genomic applications. He also served as director of neuroscience at Oncogene Research Products, where he developed a life science portfolio consisting of neurochemical and immunological products. Dr. Hanlon received his Ph.D. from the University of Illinois, Urbana-Champaign, and was a postdoctoral research fellow at Harvard University and the University of California, San Diego. He obtained a B.S. degree in biochemistry from Worcester Polytechnic Institute.

Rabih E. Jabbour, Ph.D., holds a Ph.D. in bioanalytical chemistry with more than 10 years of research experience in academia and industry in the field of the chemistry and biology of microorganisms that are of vital interest to the Department of Defense (DoD). He is leading the develop-

ment of the automated sample-processing system for the extraction and preconcentration of biological biomarkers for the detection and identification of microbes down to the strain level using mass spectrometry proteomics technology. He has contributed to various DoD projects, such as water monitoring and microbial fate using Raman imaging for the Joint Service Agent Water Monitor, toxin fate by liquid chromatography–mass spectrometry, and microbial mapping by matrix-assisted laser desorption/ionization mass spectrometry for Environmental Protection Agency projects. He has collaborated with government national laboratories and academic institutions, including the Naval Medical Research Center, Institute of Medicine, the wound infections division at Walter Reed National Medical Military Center, Johns Hopkins University, Duke University, the University of Southern California, and the University of San Francisco. He was awarded the Defense Threat Reduction Agency's Best Basic Research Award and Science Applications International Corporation's Excellence in Science Award. He serves on the editorial board for the *Journal of Integrated OMICS*, is a reviewer for the *Journal of Analytical Chemistry*, the *Journal of Proteome Research*, and *PLoS ONE* in the areas of microbial proteomics and genomics. He has published more than 25 peer-reviewed articles and proceedings in the fields of chemistry, biochemistry, spectroscopy, molecular biology, bacteriology, and protein chemistry.

Rudolph Johnson, Ph.D., is the acting manager of the Emergency Response Branch (ERB) at the Division of Laboratory Sciences, National Center for Environmental Health of the Centers for Disease Control and Prevention (CDC). Dr. Johnson oversees the development of diagnostic methods for quantifying human exposure to chemical agents. Common chemical agents measured include traditional chemical warfare agents, such as mustard gases and nerve agents, as well as selected plant and marine toxins. Dr. Johnson also supervises emergency laboratory support for human exposures to chemical agents. ERB provides laboratory support to identify the causative chemical agent following a suspected human exposure, and it conducts the Division of Laboratory Science's rapid toxic screen. ERB maintains readiness through routine exercises, proficiency testing, and Clinical Laboratory Improvement Act audits. Dr. Johnson received his Ph.D. in chemistry from Purdue University under Dr. Graham Cooks, an award-winning professor and researcher in mass spectrometry. His thesis was on the development of online mass spectrometry using membrane-introduction mass spectrometry. Dr. Johnson

received his undergraduate degree in laboratory robotics from Virginia Military Institute. Prior to working at CDC, Dr. Johnson worked at BASF Corporation, focusing on multidisciplinary projects involving small-molecule synthesis and product development, specifically, dispersions, coatings, and pharmaceutical products.

Stevan Jovanovich, Ph.D., is the chief technology officer of IntegenX Inc., a company he cofounded in 2003 to develop integrated sample preparation systems and sample-to-answer systems for the life sciences. At IntegenX he has raised more than $100 million in equity funding and $30 million in grants and contracts focused on advanced genomic systems for rapid human identification, DNA sequencing, and biodefense. Before co-founding IntegenX, Dr. Jovanovich was vice president of global research at Amersham Biosciences, where he led a team of 120 scientists and engineers and drove multiple business projects across seven international sites. Prior positions include science director at Amersham Biosciences Sunnyvale, manager of advanced research at Molecular Dynamics, staff scientist at Lawrence Berkeley National Laboratory, director of microbiology at AbTox Inc., founder of Molecular Solutions, and applications programmer at the Institute for Defense Analyses. He is the author of more than 20 papers and reviews, and an inventor on more than 70 patents, and has automated life science workflows since 1987. He holds bachelor of science degrees in physics and life sciences from the Massachusetts Institute of Technology, a Ph.D. in microbiology with a minor in molecular biology from University of California, Davis, and a certificate in object-oriented programming from the University of California, Santa Cruz, extension.

Robert Kadlec, M.D., M.T.M.&H., M.A., is a consultant at RPK Consulting LLC. Previously he was vice president, global public sector, at PRTM Management Consultants. He formerly served as special assistant to the President for homeland security and senior director for biological defense policy in the White House Homeland Security Council. Previously he served as staff director for the Senate Subcommittee on Bioterrorism and Public Health, where he oversaw the drafting of the Pandemic and All-Hazards Preparedness Act (P.L. 109-417). The law, signed by President George W. Bush on December 19, 2006, improved the functioning of Project BioShield of 2004 and reauthorized the Bioterrorism Preparedness Act of 2002. Before that, from February 2002 until March 2005, he served as director for biodefense preparedness and response at

the White House Homeland Security Council, where he was responsible for coordinating medical issues pertaining to the threat of bioterrorism with the National Security Council and the Federal Interagency. He conducted the BioDefense End-to-End Assessment and was instrumental in drafting Homeland Security Presidential Directive 10, the National BioDefense Policy for the 21st Century. In his military career, he was assigned to the Joint Special Operations Command at Fort Bragg, North Carolina, and the 16th Special Operations Wing at Hurlburt Field, Florida. He also served in senior advisory roles in the Office of the Secretary of Defense for Policy and the Central Intelligence Agency. Dr. Kadlec holds an M.D. and an M.T.M.&H. (masters in tropical medicine and hygiene) from the Uniformed Services University of the Health Sciences; an M.A. in national security studies from Georgetown University; and a B.S. from the U.S. Air Force Academy. He is board certified in aerospace and preventive medicine. He is a graduate of the Air War College.

Ivor Knight, Ph.D., is senior vice president, chief technology officer, and board member at Canon U.S. Life Sciences, Inc., where he is developing genetic diagnostic systems for Canon's entry into the health care business. Previously, Dr. Knight was a professor of biology at James Madison University, where he conducted an externally funded research program on genetic detection technologies, taught in multiple departments, and led curriculum development initiatives in molecular biology and biotechnology. He has more than two decades of experience in molecular genetic research and the development of nucleic acid–based diagnostic systems. Dr. Knight has also served as a food safety advisor to the U.S. Agency for International Development and worked for the U.S. Department of Agriculture's Foreign Agriculture Service, delivering programs and advising governments on food safety in 20 countries. Dr. Knight is a fellow of the American Association for the Advancement of Science and he received his B.S. from West Virginia University and his Ph.D. from the University of Maryland, College Park.

Charles E. Kolb, Ph.D., is the president and chief executive officer of Aerodyne Research, Inc. He joined Aerodyne as a Senior Research Scientist in 1971. At Aerodyne his personal areas of research have included atmospheric and environmental chemistry, combustion chemistry, chemical lasers, and the chemical physics of rocket and aircraft exhaust plumes. He is the author or coauthor of more than 200 archival publications in these fields. In the area of atmospheric and environmental chem-

istry, Dr. Kolb initiated Aerodyne's programs for the identification and quantification of sources and sinks of trace atmospheric gases and aerosols involved in regional and global pollution problems as well as the development of spectral sensing techniques to quantify soil pollutants. Specific atmospheric instrumentation developments include innovative tunable infrared laser differential absorption spectrometers for both remote, open path and in situ trace gas measurements and aerosol mass spectrometers for real-time analysis of airborne particle concentrations and chemical compositions as a function of particle size. These instruments have been deployed worldwide on numerous research aircraft, ships, and mobile vans and at fixed field measurements sites during atmospheric field measurement campaigns addressing air pollution and climate change issues. He has also motivated and designed chemical kinetic and molecular spectroscopy laboratory programs that provide the gas phase and gas/surface kinetic rate parameters required for atmospheric modeling as well as the quantitative spectroscopic parameters needed to design in situ measurements of trace species important in tropospheric, stratospheric, and mesospheric photochemistry. He has developed and applied models of aircraft and rocket exhaust plume/wake chemical kinetics, condensation physics, and dispersion processes critical to the systematic assessment of the impact of aerospace systems on the chemical structure of the upper troposphere and stratosphere. Dr. Kolb has been a member of numerous government and National Academy of Sciences/National Research Council boards and committees dealing with atmospheric and environmental science issues and was recognized as a national associate of the National Academies in 2003 and elected to the National Academy of Engineering in 2013. He has served as chair of the Heterogeneous Processes Subpanel of the National Aeronautics and space Administration's Panel for Chemical Kinetics and Photochemical Data Evaluation since 1991. He also received the 1997 Award for Creative Advances in Environmental Science and Technology from the American Chemical Society. He has been elected a fellow of the American Chemical Society, the American Physical Society, the Optical Society of America, the American Geophysical Union, and the American Association for the Advancement of Science and has served as the atmospheric sciences editor of the journal *Geophysics Research Letters* (1995–1999) and on the editorial advisory board of *Environmental Science & Technology* (2011–present). Dr. Kolb received his Ph.D. and M.S. degree in physical chemistry from Princeton University and a B.S. degree in chemistry from Massachusetts Institute of Technology.

Beth Maldin Morgenthau, M.P.H., is the assistant commissioner for the Bureau of Policy, Community Resilience and Response within the Office of Emergency Preparedness and Response (OEPR) at the New York City Department of Health and Mental Hygiene. In this capacity she is responsible for policy, strategic initiatives, community resilience and vulnerable population planning, interagency coordination, mass prophylaxis planning, Biowatch coordination, and the New York City Medical Reserve Corps. Ms. Maldin also plays a critical role in the agency's 24/7 response to public health emergencies. Her previous experiences include serving as executive director of policy, evaluation, and special projects within OEPR and deputy director of the Bureau of Emergency Management within Disease Control. Between October 2004 and August 2007, Ms. Maldin was an associate at the Center for Health Security of the University of Pittsburgh Medical Center, where she researched, evaluated, and analyzed national, state, and local policies and practices in the area of biosecurity. She has direct operational experience in emergency preparedness and response. She played a key role in the New York City Health Department's responses to hurricanes Irene and Sandy in 2011 and 2012, as well as its response to H1N1 in the spring and fall of 2009. She also participated in the agency's response to the 1999 West Nile virus outbreak, the September 11, 2001, attack on the World Trade Center, and the anthrax letter attacks in fall 2001. Ms. Maldin earned an M.P.H. in policy and management from the Columbia University School of Public Health and a B.S. in anthropology and human biology from Emory University. She is also a certified project management professional.

Raymond Mariella, Jr., Ph.D., received his B.A. from Rice University, where he graduated magna cum laude with a triple major in mathematics, chemistry, and chemical engineering, with research under Robert F. Curl, Jr. He received his A.M. and Ph.D. in physical chemistry from Harvard University under Dudley Herschbach and William Klemperer. He taught physical chemistry at Harvard and was a visiting scientist in the physics department at the Massachusetts Institute of Technology. He spent 10 years at the Allied-Signal Corporate Research Center, in Morris Township, New Jersey. At Lawrence Livermore National Laboratory (LLNL), he has served as the project leader for III-V semiconductor devices, team leader for bioinstrumentation, and the director of the Center for Micro and Nano Technology. He currently supports all LLNL missions as a senior scientist. Dr. Mariella is also the co-inventor on a patent of a sleeve-based thermal cycler, licensed by Cepheid, Northrop Grumman,

and MFSI (expiration in 2016). He has served as the chairman of the Science and Technology Working Group for the National Aeronautics and Space Administration's (NASA's) Advanced Environmental Monitoring and Control Program and has chaired NASA solicitation review panels, and he has served on National Science Foundation (NSF) review panels on nanoscience and nanotechnology and the external advisory committee for the NSF Center for Microbial Oceanography: Research and Education. Dr. Mariella has also served on the National Academies/National Research Council (NRC) Committee on Materials and Manufacturing Processes and the NRC committee to review NASA's Exploration Technology Development Programs.

Suzet M. McKinney, Dr.P.H., currently serves as deputy commissioner of the Bureau of Public Health Preparedness and Emergency Response at the Chicago Department of Public Health (CDPH), where she oversees the emergency preparedness efforts for the department, coordinating those efforts within the larger spectrum of the City of Chicago's public safety activities. Dr. McKinney also oversees the CDPH Division of Women and Children's Health and is the former senior advisor for public health and preparedness at the Tauri Group, where she provided strategic and analytical consulting services to the Department of Homeland Security's BioWatch Program, including creative, responsive, and operationally based problem solving for public health, emergency preparedness, and homeland security issues, specifically chemical and biological early detection systems and the implementation of those systems at the state and local level. She serves as incident commander for CDPH and is a member of Chicago's incident management team. In academia, Dr. McKinney serves as adjunct assistant professor of community health sciences at the University of Illinois at Chicago School of Public Health and as the coordinator of the school's online emergency preparedness certificate program. She also serves as a mentor for the Biomedical Sciences Careers Project at Harvard University as well as for the National Preparedness Leadership Initiative Executive Education Program at Harvard University. Dr. McKinney holds a doctorate degree from the University of Illinois at Chicago School of Public Health, a master of public health degree from Benedictine University in Lisle, Illinois, and a bachelor of arts in biology from Brandeis University.

M. Allen Northrup, Ph.D., is the founder of Microfluidic Systems, Inc. (MFSI) and a cofounder of Cepheid. He is currently the science advisor

to three startup companies in the United States and Europe. Dr. Northrup received his Ph.D. in biomedical engineering from the University of California, Davis (UCD), in 1991. During his early years at UCD, Dr. Northrup worked for Finnigan Corporation, a manufacturer of gas chromatograph/mass spectrometers. As a postdoctoral fellow and engineer at Lawrence Livermore National Laboratory (LLNL) and as a visiting scholar at the University of California, Berkeley, he was the first to demonstrate the polymerase chain reaction in a micromachined silicon chip. He also invented and co-developed microactuators and microactuator materials at LLNL. As chief technology officer at Cepheid, he was on the initial public offering team in 2000, and as chief executive officer of MFSI, Dr. Northrup sold MFSI to Positive ID Corporation in 2011. One of his inventions at Cepheid is the basis for detection in the U.S. Postal Service's Biological Detection System. Dr. Northrup has 51 patents, 40 publications, and several engineering and entrepreneurial business awards. Dr. Northrup has been the principal investigator on more than $85 million of funding from U.S. government agencies, such as the Department of Homeland Security (DHS), and the Department of Defense (DoD). He co-invented numerous bioanalytical systems, including the commercial Cepheid systems SmartCycler and GenXpert. Dr. Northrup has delivered numerous bio-analytical systems to DHS, DoD, and the United Kingdom. He is a member of the National Academy of Engineering and a fellow of the American Institute of Biological and Medical Engineers.

Ted Olsen is president and chief executive officer of PathSensors, Inc., a biotechnology company based in Baltimore, Maryland. Mr. Olsen is an operations executive with a broad set of experiences in new product development and manufacturing. He has been instrumental in building and expanding high-technology manufacturing companies around the world. Among those are: Innovative Biosensors, Inc.; Corvis Corporation; Optical Fibres; Seicor GmbH; Optical Waveguides Australia; Corning Asahi Video Products; and Corning, Incorporated's Photonic Technology Division. He is a member of the State of Maryland Life Science Advisory Board and a graduate from the Catholic University of America.

William O'Neill, Ph.D., is the development program manager and project engineer for the U.S. Postal Service Biohazard Detection System a fully autonomous biothreat screening system. The system was the first Safety Act certified system and is recognized by the Centers for Disease Control and Prevention as a public health actionable system. The system

has been operational for a decade with no false-positive results in more than 11.5 million polymerase chain reaction tests.

Zheng Ouyang, Ph.D., obtained his bachelor's and master's degrees in electric engineering from Tsinghua University, his second master's degree in physical chemistry from West Virginia University, and his Ph.D. in analytical chemistry from Purdue University. He currently is an associate professor in the Weldon School of Biomedical Engineering at Purdue University. His research interests include the mass spectrometry instrumentation and application in biomedical diagnosis. His research is supported by the National Science Foundation (NSF), the National Institutes of Health, the National Aeronautics and Space Administration, and the Department of Defense. He also receives funding from bioMérieux, which produces automated mass spectrometry systems for use in clinical laboratories. Dr. Ouyang has published more than 100 papers and has received the NSF Early Career Award, Coulter Foundation Early Career Award, American Society for Mass Spectrometry Research Award, and the International Foundation of Mass Spectrometry Curt Brunnee Award.

Erica Pan, M.D., M.P.H., is a deputy health officer and the director of the Division of Communicable Disease Control and Prevention in the Alameda County Public Health Department. She joined Alameda County Public Health Department in November 2011 after serving as the director of the Public Health Emergency Preparedness and Response Section and deputy health officer at the San Francisco Department of Public Health. Prior to this position, from 2004 to 2011 she was the director of the Bioterrorism and Infectious Disease Emergencies Unit in the Communicable Disease Control and Prevention Section at the San Francisco Department of Public Health. Dr. Pan served as a participant in the American Academy of Pediatrics (AAP)/Centers for Disease Control and Prevention (CDC) Anthrax Disaster Preparedness and Planning: Pediatric Clinical Guidance Meeting and the AAP/CDC Pediatric Anthrax Vaccines Workgroup in 2012, as a participant in the National Biodefense Science Board Anthrax Vaccine Working Group Medical Countermeasures for Children—Anthrax Vaccine Workshop in 2011, and as a member of the National Biosurveillance Advisory Committee (a subcommittee of the CDC Director's Advisory Committee) from 2008 to 2010. She is also an associate clinical professor in the Department of Pediatrics, Division of Pediatric Infectious Diseases, at the University of California, San Francisco (UCSF). She has maintained her clinical work attending at UCSF

for pediatric infectious diseases, at San Francisco General Hospital in the general pediatrics clinic, and at Children's Hospital Oakland in infectious diseases. Her previous training includes completion of a pediatric residency, chief residency, and pediatric infectious disease, and traineeship in AIDS prevention studies fellowships at UCSF. She is board certified in both pediatric infectious diseases and pediatrics. She received her M.D. and M.P.H. degrees from Tufts University School of Medicine and completed her undergraduate education at Stanford University.

David Persse, M.D., has been the physician director of emergency medical services for the City of Houston since 1996 and for the Public Health Authority since 2004. A graduate of the Georgetown University School of Medicine, he completed his emergency medicine residency at Harbor–UCLA (University of California, Los Angeles) Medical Center and completed two fellowships, one at Ohio State University and one at Baylor College of Medicine. He is now on faculty at both the Baylor College of Medicine and the University of Texas Medical School–Houston. He is also a tactical physician with the Houston Police S.W.A.T. team.

Sally Phillips, R.N., Ph.D., serves as the principal deputy assistant secretary (acting) within the Department of Homeland Security's (DHS's) Office of Health Affairs (OHA). Dr. Phillips joined DHS in August 2010 and served as the deputy director of the Health Threats Resilience Division until March 2012. Dr. Phillips provides leadership and direction to five major programmatic areas within OHA: biological and chemical defense; food, agriculture, and veterinary; planning and exercises; health incidence surveillance; and state and local initiatives. Dr. Phillips came to OHA from the Agency for Healthcare Research and Quality (AHRQ), where she served as director of the Public Health Emergency Preparedness Research Program. Dr. Phillips joined AHRQ in fall 2001 as a senior nurse scholar, where she managed a portfolio ranging from bioterrorism preparedness to multidisciplinary safety education and related health care workforce initiatives. Dr. Phillips was appointed director of the Bioterrorism Preparedness Research Program (now the Public Health Emergency Preparedness Research Program) in 2002. In July 2009, Dr. Phillips joined the Assistant Secretary for Preparedness and Response (ASPR) as a senior advisor. There she was involved in policy issues, primarily supporting the H1N1 Task Force by addressing medical surge capacity and policies related to health care systems' preparedness and response to H1N1. After completing her ASPR detail in January 2010,

she returned to AHRQ to continue serving as the director of the Public Health Emergency Preparedness Research Program. Dr. Phillips is a leader in health systems' surge capacity and in emergency preparedness. In her role at AHRQ, she served on numerous agency and department workgroups concerned with public health and medical response, as well as homeland security preparedness and response initiatives. She is an accomplished author, consultant, and speaker on public health and medical preparedness and response research initiatives. She also has additional expertise in health professional education and professional practice policy. Prior to joining AHRQ, Dr. Phillips was a Robert Wood Johnson health policy fellow and health policy analyst for Senator Tom Harkin for 2 years. She has also had a distinguished academic career in the Schools of Nursing and Medicine at the University of Colorado Health Sciences Center. Dr. Phillips received a bachelor's degree from Ohio State University, a master's degree from the University of Colorado, and a doctorate from Case Western Reserve University. Her primary area of clinical practice is the care of women, infants, and children, with a specialty in the care of high-risk neonates.

John Plante, J.D., is the senior manager of emergency preparedness for the Chicago Transit Authority (CTA). He has more than 34 years of experience with the CTA and began working for the CTA as a trial attorney. From there he moved to trial supervision and then managing attorney. He created and managed the first litigation team in the CTA law department assigned to handle the litigation of major injuries and fatalities. Recognizing that litigation results from the intended or unintended actions of others, Mr. Plante expanded his competency well beyond litigation to the areas of claims, risk, safety, and, ultimately, to emergency preparedness and management. Following the events of September 11, 2001, the increasing focus of Mr. Plantes' activities became external risk—natural, technological, and societal. As a strong advocate of emergency preparedness, Mr. Plante was responsible for the recognition of emergency preparedness and management as a separate and distinct discipline at the CTA. Mr. Plante, who is accredited by the State of Illinois as a professional emergency manager, serves as the CTA representative to local, state, and national emergency and security departments and agencies as well as a number of emergency preparedness working groups.

Donald Prosnitz, Ph.D., joined the RAND Corporation in September 2007 as a senior principal researcher. His studies at RAND concentrate on the utilization of technology to solve national and homeland security issues. Dr. Prosnitz was previously the deputy associate director (programs) for Nonproliferation, Homeland, and International Security at Lawrence Livermore National Laboratory, where he was responsible for overseeing all of the directorate's technical programs. He received his B.S. from Yale University and his Ph.D. in physics from the Massachusetts Institute of Technology. He then spent 2 years as an assistant professor in the Engineering and Applied Science Department at Yale University before joining Lawrence Livermore National Laboratory as an experimental laser physicist. During the next three decades he conducted research on lasers, particle accelerators, high-power microwaves, free-electron lasers, and remote sensing and managed the design, construction, and operation of numerous research facilities. In 1990 he was awarded the U.S. Particle Accelerator Award for Achievement in Accelerator Physics and Technology. In 1999 Dr. Prosnitz was named the first chief science and technology advisor for the Department of Justice (DOJ) by Attorney General Janet Reno. In this newly created position he was responsible for coordinating technology policy among DOJ's component agencies and with state and local law enforcement entities on science and technology projects and programs. In 2002 he was named a fellow of the American Physical Society. He is currently a member of the National Academy of Sciences' Board on Chemical Sciences and Technology.

Gary Resnick, Ph.D., is an independent consultant and guest scientist at Los Alamos National Laboratory. He is a systems thinker with extensive experience and successes in organizational analysis and performance as well as strategic planning and implementation. He is an internationally recognized scientist in the area of chemical and biological defense with extensive technical leadership/management experience. His scientific and technical accomplishments encompass all aspects of research, development, and testing of chemical and biological warfare agents and defense systems. In addition, he has been an active member of the interagency and international chemical and biological weapons arms control communities.

Jeffrey W. Runge, M.D., is a principal at the Chertoff Group, a firm providing business risk management and security sector advisory services, and president of Biologue, Inc., a consulting firm specializing in

biodefense, medical preparedness, and injury prevention and control. Dr. Runge serves on the Board of Directors of PharmAthene, Inc., a developer of next-generation anthrax vaccine and chemical nerve agent countermeasures. He is also an adjunct professor in the School of Medicine at the University of North Carolina at Chapel Hill. From 2005 to 2008, Dr. Runge served as the Department of Homeland Security's (DHS's) first chief medical officer and led the reorganization of biodefense operations into a new Office of Health Affairs (OHA). OHA acts as the principal advisor to all DHS component agencies on medical, biodefense, and workforce health issues. From 1984 to 2001, he practiced and taught emergency medicine at Carolinas Medical Center in Charlotte. His primary research areas were in injury prevention, trauma care, and emergency service delivery. His leadership and innovation in injury prevention programs brought him to Washington as the head of the National Highway Traffic Safety Administration (NHTSA) in 2001. At NHTSA he instituted programs, regulations, and policies that led to the first absolute declines in U.S. motor vehicle deaths in almost a decade and the lowest highway fatality rate in history. Dr. Runge is board-certified in emergency medicine and has published more than 60 articles in medical literature in the fields of emergency medicine, traffic injury control, and medical preparedness. Dr. Runge is a graduate of the University of the South in Sewanee, Tennessee, and received his medical degree from the Medical University of South Carolina.

R. Paul Schaudies, Ph.D., is an internationally recognized expert in biotechnology and nanotechnology. Dr. Schaudies is the chief executive officer of GenArraytion, Inc., located in Rockville, Maryland. GenArraytion is a medical diagnostics company combining bioinformatics with array technology for the simultaneous identification and characterization of microorganisms. He has served on 12 National Research Council (NRC) committees on varied technology areas. In addition to his NRC service, Dr. Schaudies has served on advisory committees for several U.S. government agencies, including the Defense Advanced Research Projects Agency and the Department of Energy. He has served as chairman for various conferences, including several Gordon Research Conferences. Dr. Schaudies served as a science advisor to the Environmental Protection Agency On-Scene Coordinator, the sergeant at arms of the Senate and House of Representatives, and incident commander at the 2001 anthrax incident in Washington, DC. Dr. Schaudies has more than 20 years of experience in the biomedical research community, 4 years in the de-

fense and intelligence community, and 9 years as a corporate management executive at Science Applications International Corporation. He spent 13 years as an active duty U.S. Army officer and retired as a lieutenant colonel in the U.S. Army Reserve. While on active duty, Dr. Schaudies served as program manager for biological and chemical defense research at the Defense Intelligence Agency and served as a United Nations Special Commission inspector in Iraq. Other assignments included senior researcher at the Walter Reed Army Institute of Research and chief of the General Support Laboratory in the Department of Clinical Investigation at Walter Reed Army Medical Center. Dr. Schaudies holds a bachelor's degree in chemistry from Wake Forest. He earned his Ph.D. in biochemistry at the Temple School of Medicine and was awarded a visiting scientist position with Stewart Aaronson in the Laboratory of Cellular and Molecular Biology at the National Institutes of Health National Cancer Institute.

Jeffery A. Schloss, Ph.D., is director of the Division of Genome Sciences and also program director for technology development coordination in the extramural research program at the National Human Genome Research Institute (NHGRI), a component of the National Institutes of Health (NIH). At NHGRI, he serves the extramural program and Office of the Director as a resource on genome technology development issues. He leads the team that launched and manages the centers of excellence in genomic science, and he initiated a program to foster effective collaborations to validate new sequencing technologies for use in high-throughput laboratories. He implemented and manages the institute's program to develop technologies with which to sequence entire human genomes at a cost of $1,000. He previously served the NHGRI as program director for large-scale genetic mapping, physical mapping, and DNA sequencing projects. Dr. Schloss represented NHGRI on the NIH Bioengineering Consortium, BECON, established in 1997 to foster support for bioengineering research, and served as the chair of BECON from 2001 to 2004. He represented the NIH on the National Science and Technology Council's Subcommittee on Nanoscale Science, Engineering and Technology Planning for the National Nanotechnology Initiative. He also cochairs the working group for the NIH Nanomedicine Roadmap Initiative. Dr. Schloss served on the biology faculty at the University of Kentucky. He earned a B.S. degree with honors from Case Western Reserve University and a Ph.D. in cell biology from Carnegie Mellon University, and he conducted postdoctoral research at Yale University.

Umair A. Shah, M.D., M.P.H., was appointed in May 2013 as the executive director of Harris County Public Health and Environmental Services (HCPHES) and the local health authority for Harris County, Texas, the third most populous county in the United States. Previously, Dr. Shah had served since 2004 as HCPHES deputy director and also as its director of disease control and clinical prevention. Prior to joining HCPHES he was an emergency department physician at Houston's Michael E. DeBakey VA Medical Center and chief medical officer at the Galveston County Health District. He earned a B.A. in philosophy from Vanderbilt University, an M.D. from the University of Toledo Health Science Center, and a primary care/general medicine fellowship and his M.P.H. in management and policy sciences at the University of Texas Health Science Center before completing his internal medicine residency. His focus areas include population health, wellness, and prevention; health care management; global and refugee health; health equity; and community engagement. His large-scale emergency response roles have included responses to Tropical Storm Allison; Hurricanes Ike, Katrina, and Rita; novel H1N1; and the earthquakes in Kashmir and Haiti. In addition to completing an international health policy internship at the World Health Organization in Geneva, he has provided leadership through the American Public Health Association, the Centers for Disease Control and Prevention, the Institute of Medicine of the National Academies, and the National Association of County and City Health Officials. He remains engaged in clinical patient care and academic teaching and is actively involved in the local Harris County community.

Thomas Slezak, M.S., is a computer scientist who has been involved with bioinformatics at Lawrence Livermore National Laboratory (LLNL) since 1978. Mr. Slezak is currently the associate program leader for informatics for the global security program efforts at LLNL. He was part of the Human Genome Program for 14 years and part of the team that developed the BASIS and BioWatch systems. Mr. Slezak has chaired or served on multiple advisory boards, including the U.S. rice genome sequencing and annotation projects, mouse and maize genetics databases, and a National Institute of Allergy and Infectious Diseases sequencing center contract renewal. He recently has served on two National Academy panels (Select Agent Science and Core Competencies of DoD Laboratories) and currently serves on the National Academy of Sciences DoD Standing Committee on Biodefense programs. From 2010 to 2012 Mr. Slezak served on the National Biosurveillance Advisory Subcommittee.

He also cochaired a blue ribbon panel for the Centers for Disease Control and Prevention that examined the current state of bioinformatics at that agency and recommended paths for improvement. Mr. Slezak's team has extensive experience in building computational systems to design pathogen detection signatures and analyze genomic sequence as well as in building fielded systems that utilize these capabilities.

Sandra C. Smole, Ph.D., is director of the Division of Molecular Diagnostics and Virology in the Bureau of Laboratory Sciences at the Massachusetts Department of Public Health (MDPH). In this position she is responsible for overseeing six laboratories, including the BioWatch laboratory since 2006. Her laboratory plays as active role in field response coordination for the Massachusetts BioWatch jurisdiction. She has participated in the BioWatch program since 2003. She currently serves on the Association of Public Health Laboratories (APHL) infectious diseases committee and continues to serve on subcommittees such as Advanced Molecular Detection. She has served as a member of the APHL influenza subcommittee, and her laboratory actively works with the Centers for Disease Control and Prevention's (CDC's) influenza branch on validating new technologies for influenza preparedness. Previously, Dr. Smole was a CDC/APHL emerging infectious diseases research fellow spending time at both the CDC's Bioterrorism Rapid Response and Advanced Technology Laboratory and at the MDPH. Prior to this fellowship she completed her postdoctoral training in molecular epidemiology from Boston University School of Medicine's Department of Medicine while at the Boston Veterans Administration Medical Center. Dr. Smole received her B.S. in biology from the University of Portland and her Ph.D. in microbiology and immunology from Baylor College of Medicine in Houston, Texas.

A. Peter Snyder, Ph.D., is a research chemist who is currently working on a number of scientific projects. His work with Hampton University and Brimrose focuses on laser-induced breakdown spectroscopy and laser-induced thermal emission to show that these methods can provide infrared signatures of various biochemical and bacterial compounds. Dr. Snyder is exploring Raman chemical imaging microspectroscopy (visual and multivariate data analysis techniques) to obtain biological signatures for bacteria and protein toxin differentiation in water matrices in collaboration with Science Applications International Corporation (SAIC) and ChemImage, Inc. He is also collaborating with SAIC and the Science and

Technology Corporation (STC) for studies in the differentiation and identification of single bacteria and mixtures of two to eight different bacteria in water buffer suspensions. Proteomics-electrospray with liquid chromatography–mass spectroscopy technologies is the analytical system used. Further, sophisticated data analysis and reduction software developed by SAIC and STC are being used for the bacterial identification. The mass spectroscopy proteomics approach proved capable of identifying and classifying organisms within a microbial mixture. Additionally, Dr. Snyder is writing a series of papers that deal with using receiver operating characteristic curve mathematics and novel Gaussian univariate statistics that have not been utilized before for differentiation of multiple cases and many, many variables. Finally, Dr. Snyder is researching proteomics differentiation of biological substances by a combination of mass spectrometry and Raman spectroscopy. The ultimate goal is possible therapeutic ramifications of bacterial disease by determining the enzymes responsible for being expressed during the 1-day "growth" period in nutrient-free water.

Colin Stimmler, M.A., is the director of the BioWatch program at the New York City Department of Health and Mental Hygiene (DOHMH) within the Office of Emergency Preparedness and Response in the Bureau of Policy, Community Resilience & Response. He coordinates the DOHMH BioWatch workgroup, developing tools and protocols for agency leadership and advancing various policy issues for resolution. He is also the agency's official liaison to the New York City BioWatch Stakeholder Group. He has represented the DOHMH on numerous BioWatch national workgroups and focus groups. Prior to his current position he was an emergency planner with DOHMH, working on the development of the threat response guides and the school vaccination program for the fall 2009 H1N1 campaign. And prior to that, he was an emergency planner with the New York City Department of Homeless Services, developing various aspects of New York City's Coastal Storm Plan Shelter System. Mr. Stimmler has a master's degree in international political economy and development from Fordham University and is a former Peace Corps volunteer, having spent 2 years in Nepal.

David Tilles, M.S., is responsible for Northrop Grumman's chemical, biological, radiological, nuclear, and explosive (CBRNE) detection and defense business area, which supports various government customers, including the Department of Homeland Security, the Department of De-

fense, and the U.S. Postal Service. Mr. Tilles has more than 30 years of experience in technology development, systems engineering, and program management related to automated systems and CBRNE detection. He has a B.E.S. in materials science and engineering and an M.S. in technical administration from Johns Hopkins University.

John Vitko, Jr., Ph.D., is the former director of biological and chemical countermeasures for the Science and Technology Directorate of the Department of Homeland Security (DHS). In that role, he had overall responsibility for all DHS science and technology to deter, detect, or mitigate a biological or chemical attack on the people, infrastructure, or agriculture of this nation. Prior to that, Dr. Vitko was a director of exploratory systems at Sandia National Laboratories in Livermore, California, where he had been since receiving his Ph.D. in physics from Cornell University in 1975. Trained as a solid-state physicist and spectroscopist, Dr. Vitko has conducted basic and applied research in support of defense and energy programs; led a major portion of Sandia's strategic defense programs in the 1980s; been the technical director of a multilaboratory Department of Energy (DOE) program on the use of unmanned aerospace vehicles for climate research in the 1990s; played a formative role in many advanced detection technology programs at Sandia, ranging from lidars to a hand-held suite of chromatography labs known as μChemLab; led all of Sandia's biological and chemical defense programs; served as coordinator for the detection thrust area of DOE's multi-laboratory Chemical and Biological Non-Proliferation Program and as the DOE representative to the multiagency ChemBio Detection Roadmapping Committee. In September 2002 he went on temporary assignment to Washington, DC, to help in the planning stages for the Department of Homeland Security and has subsequently joined that agency on an IPA (interagency personnel agreement) status. Dr. Vitko also chaired a National Research Council study on advanced sensors for bioagent detection. He received his Ph.D. in physics from Cornell University.

Michael V. Walter, Ph.D., is the detection branch chief and BioWatch program manager for the U.S. Department of Homeland Security's Office of Health Affairs. Dr. Walter joined the Office of Health Affairs as the BioWatch program manager in September 2009. Since joining BioWatch, Dr. Walter has instituted a robust quality assurance program. Working with the Centers for Disease Control and Prevention (CDC), he directed the transition of BioWatch sample screening from CDC poly-

merase chain reaction (PCR) assays to Department of Defense (DoD) Critical Reagent Program PCR reagents. In addition, he has overseen the successful completion of the Gen-3 Phase I Acquisition Program. He has worked to increase interoperability and partnerships with federal, state, and local BioWatch program stakeholders. He was recognized as "One of the Faces of Homeland Security" by Secretary Napolitano in 2011. Dr. Walter possesses more than 20 years' experience in microbiology/ biological warfare research. He has an extensive background in sampling and detection for aerosolized microorganisms as well as in the management and development of design, test, evaluation, and quality assurance for related systems and programs. He also has significant experience in laboratory assay development, testing, and evaluation. Prior to joining BioWatch, Dr. Walter was a staff senior scientist and headed the technology special project team for the DoD Joint Program Executive Office for Chemical and Biological Defense. He has also held positions with the Central Intelligence Agency, the Naval Surface Warfare Center, and Texaco, Inc. Dr. Walter is the recipient of eight publication and innovation awards and author of numerous scientific articles, abstracts, and patents. He received his Ph.D. in microbiology from the University of North Dakota.

Brian Young, Ph.D., is a program manager at Battelle, where he has worked for more than 25 years. He currently directs Battelle's internally funded research on the application of next-generation sequencing to forensic DNA analysis. This work focuses on developing bioinformatic workflows suitable in forensic and diagnostic applications and has resulted in the first successful method for allelo-typing microsatellites in short-read data. Previously, Dr. Young managed a number of programs in forensic analysis of environmental samples involving both biological and chemical signatures.

C

Workshop Agenda

Strategies for Cost-Effective and Flexible Biodetection Systems That Ensure Timely and Accurate Information for Public Health Officials: A Workshop

AGENDA

National Academy of Sciences Building, Room 125
2101 Constitution Avenue, NW • Washington, DC 20001

The Institute of Medicine and the National Research Council will host a 2-day workshop that will explore alternative cost-effective systems that would meet requirements for BioWatch as an automated detection system for aerosolized agents (alternative technologies for autonomous detection). Systems identified need to be capable of being deployed by the Department of Homeland Security (DHS) by 2018 and enabling day-to-day environmental surveillance that would be of value to the public health and medical community.

Workshop Objectives:

- Develop an understanding of the nature of the biothreat and the role of biodetection.

- Discuss the history of the BioWatch program and the draft request for proposals (RFP) released by DHS for alternative technologies for autonomous detection.
- Discuss the role of public health officials and laboratorians in the interpretation of BioWatch data and the information that is needed from a system for effective decision making.
- Review the current state of the art and explore the potential use of four families of biodetection technology for the BioWatch program.
- Explore how the technologies discussed might be strategically combined or deployed to optimize their contributions to an effective environmental detection capability.

DAY ONE

8:00 a.m. Breakfast available for planning committee and speakers

8:30 a.m. **Welcome, Introductions, and Meeting Objectives**

WILLIAM RAUB, *Chair, Workshop Planning Committee*
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

SALLY PHILLIPS
Principal Deputy Assistant Secretary (Acting)
Office of Health Affairs
Department of Homeland Security

SESSION I: OVERVIEW OF THE BIOWATCH PROGRAM

Session Objectives: Develop an understanding of the nature of the biothreat and the role of biodetection. Discuss the history of the BioWatch Program and the draft RFP released by the Department of Homeland Security regarding alternative technologies for autonomous detection.

8:45 a.m. **Nature of the Biothreat**

Moderator:
WILLIAM RAUB
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

ROBERT KADLEC
Consultant
RPK Consulting LLC
Former Special Assistant to the President for Homeland Security
Former Senior Director for Biological Defense Policy
White House Homeland Security Council

5 Minutes for Questions

9:05 a.m. **BioWatch Program History**

JEFFREY RUNGE
Principal
The Chertoff Group
Assistant Secretary for Health Affairs and Chief Medical Officer (2005–2008)
Department of Homeland Security

5 Minutes for Questions

9:25 a.m. **Current BioWatch Program, Technology, and Autonomous Detection**

MICHAEL V. WALTER
Detection Branch Chief
BioWatch Program Manager
Office of Health Affairs
Department of Homeland Security

20 Minutes for Discussion (specific to the BioWatch Program and alternative technologies for autonomous detection)

10:05 a.m. Break

SESSION II: PUBLIC HEALTH AND DECISION MAKERS

Session Objectives: Discuss role of public health officials and laboratorians in the interpretation of BioWatch results. What information is needed to call a BioWatch Actionable Result (BAR) (i.e., a positive result, not necessarily a threat to public health) in the current system? What information would be needed for confidence in a BAR determined using an automated detection system? Once a BAR is determined, what data are needed for analysis and to help determine if there is a threat to public health (i.e., what decisions or actions may be taken as result of a BAR)?

10:20 a.m. **Panel Discussion: BioWatch—Information for Decision Making**

Moderator:
SUZET M. MCKINNEY
Deputy Commissioner
Bureau of Public Health Preparedness and Emergency Response
Division of Women & Children's Health
Chicago Department of Public Health

Rapporteur:
BETH MALDIN MORGENTHAU
Assistant Commissioner
Bureau of Policy, Community Resilience and Response
Office of Emergency Preparedness and Response
New York City Department of Health and Mental Hygiene

Commissioned Paper Writer:
SANDRA SMOLE
Director
Division of Molecular Diagnostics and Virology
Bureau of Laboratory Sciences
Massachusetts Department of Public Health

Panel:
UMAIR A. SHAH
Executive Director
Local Health Authority
Harris County Public Health and Environmental Services

DAVID PERSSE
Emergency Medical Services Physician Director
Public Health Authority
City of Houston

ERICA PAN
Deputy Health Officer and Director
Division of Communicable Disease Control & Prevention
Alameda County Public Health Department

COLIN STIMMLER
Director of the BioWatch Program
New York Department of Health and Mental Hygiene

JOHN PLANTE
Senior Manager of Emergency Preparedness
Chicago Transit Authority

12:15 p.m. Break for Lunch (lunch available for planning committee and speakers)

SESSION III: REVIEW OF POTENTIAL TECHNOLOGIES FOR THE BIOWATCH PROGRAM

Session Objectives: Explore the potential use of four families of technology for the BioWatch Program: (1) nucleic acid signatures, (2) immunoassays and protein signatures, (3) genomic sequencing, and (4) mass spectrometry.

1:00 p.m. **Panel: State of the Art for Autonomous Detection Systems Using Nucleic Acid Signatures**

Moderator:
JOHN VITKO
Rector
St. Luke Church
Director of Biological and Chemical Countermeasures for Science and Technology Directorate (Retired)
Department of Homeland Security

Rapporteur:
WILLIAM O'NEILL
Development Program Manager and Project Engineer
Biohazard Detection System
U.S. Postal Service

Commissioned Paper Writer:
RAYMOND P. MARIELLA, JR.
Senior Scientist
Lawrence Livermore National Laboratory

Panel:
DAVID TILLES
Vice President
CBRNE Defense
Northrop Grumman

M. ALLEN NORTHRUP
Chief Executive Officer
Northrup Consulting Group

STEVAN JOVANOVICH
Co-Founder and Chief Technology Officer
IntegenX, Inc.

IVOR KNIGHT
Senior Vice President and Chief Technology Officer
Canon U.S. Life Sciences, Inc.

3:00 p.m. Break

3:15 p.m. **Panel: State of the Art for Autonomous Detection Systems Using Immunoassays and Protein Signatures**

Moderator:
WILLIAM RAUB, *Chair, Workshop Planning Committee*
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

Rapporteur:
THOMAS SLEZAK
Associate Program Leader
Informatics for the Global Security Program
Lawrence Livermore National Laboratory

Commissioned Paper Writer:
R. PAUL SCHAUDIES
Chief Executive Officer
GenArraytion, Inc.

Panel:
AMY ALTMAN
Vice President
Biodefense and Food Safety
Luminex Corporation

TED OLSEN
Chief Executive Officer and President
PathSensors, Inc.

ANDREW BARTKO
Principal Scientist
Battelle Memorial Institute

DAVID HANLON
Director
Business Development and Strategic Collaborations
Quanterix

5:00 p.m. **Concluding Remarks**

WILLIAM RAUB, *Chair, Workshop Planning Committee*
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

5:30 p.m. **Adjurn**

Room will be open until 6:30 and participants are encouraged to continue conversations.

DAY TWO

8:15 a.m. Breakfast available for planning committee and speakers

8:45 a.m. **Welcome and Overview**

WILLIAM RAUB, *Chair, Workshop Planning Committee*
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

SESSION III (CONT.): REVIEW OF POTENTIAL TECHNOLOGIES FOR THE BIOWATCH PROGRAM

Session Objectives (cont.): Explore the potential use of four families of technology for the BioWatch Program: (1) nucleic acid signatures, (2) immunoassays and protein signatures, (3) genomic sequencing, and (4) mass spectrometry.

9:00 a.m. **Panel: State of the Art for Autonomous Detection Systems Using Genomic Sequencing**

Moderator:
RITA COLWELL
Distinguished University Professor
University of Maryland
Johns Hopkins University
School of Public Health

Rapporteur:
ERIC EISENSTADT
Independent Technical Consultant

Commissioned Paper Writers:
CHRIS DETTER
Bio-Threat/Bio-Defense Program Director
Emerging Threats Program Office
Global Security and Bioscience Division
Los Alamos National Laboratory

GARY RESNICK
Independent Consultant
Guest Scientist
Los Alamos National Laboratory

Panel:
JEFFREY SCHLOSS
Director, Division of Genome Sciences
Program Director, Technology Development and Coordination
Extramural Research Program
National Human Genome Research Institute
National Institutes of Health

THOMAS CEBULA
Chief Technical Officer
CosmosID™
Visiting Professor
Johns Hopkins University

BRIAN YOUNG
Program Manager
Battelle Memorial Institute

10:30 a.m. Break

10:45 a.m. **Panel: State of the Art for Autonomous Detection Systems Using Mass Spectrometry**

Moderator:
DONALD PROSNITZ
Independent Consultant

Rapporteur:
CHARLES KOLB
President and Chief Executive Officer
Aerodyne Research, Inc.

Commissioned Paper Writers:
A. PETER SNYDER
Private Citizen

RABIH JABBOUR
Private Citizen

Panel:
RUDOLPH JOHNSON
Acting Manager
Emergency Response Branch
Division of Laboratory Sciences
National Center for Environmental Health
Centers for Disease Control and Prevention

ERIC E. GARD
Scientist
Defense Biology Division
Lawrence Livermore National Laboratory

ZHENG OUYANG
Associate Professor
Weldon School of Biomedical Engineering
Purdue University

SESSION IV: TECHNOLOGIES AS PART OF COMPLETE ENVIRONMENTAL DETECTION CAPABILITY

Session Objectives: Review the themes and key points from previous panel discussions. Explore how the technologies discussed might be strategically combined or deployed to optimize their contributions to an effective environmental detection capability. Additional consideration of technologies and how they might fit together as whole system (e.g., modular, hybrid, etc.).

1:30 p.m. **Panel of Rapporteurs**

Moderator:
WILLIAM RAUB, *Chair, Workshop Planning Committee*
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

Panel:
BETH MALDIN MORGENTHAU
Assistant Commissioner
Bureau of Policy, Community Resilience and Response
Office of Emergency Preparedness and Response
New York City Department of Health and Mental Hygiene

SANDRA SMOLE
Director
Division of Molecular Diagnostics and Virology
Bureau of Laboratory Sciences
Massachusetts Department of Public Health

WILLIAM O'NEILL
Development Program Manager and Project Engineer
Biohazard Detection System
U.S. Postal Service

THOMAS SLEZAK
Associate Program Leader
Informatics for the Global Security Program
Lawrence Livermore National Laboratory

CHARLES KOLB
President and Chief Executive Officer
Aerodyne Research, Inc.

ERIC EISENSTADT
Independent Technical Consultant

3:00 p.m. **Concluding Remarks**

WILLIAM RAUB, *Chair, Workshop Planning Committee*
Science Advisor to the Secretary (Retired)
Department of Health and Human Services

3:30 p.m. **Adjourn**

D

Registered Attendees

Amy Altman
Luminex Corporation

Jessica Appler
Department of Homeland Security

Hazel Bailey
Government Accounting Office

Andrew Bartko
Battelle Memorial Institute

Linda Beck
Department of Homeland Security

Steven Bennett
Department of Homeland Security

Laura Biesiadecki
National Association of County and City Health Officials

Jeffrey Bigongiari
CBRNe World

Ava-Gay Blagrove
New York City Health

Cynthia Boston
PathSensors, Inc.

Debora Boyle
The Tauri Group—BioWatch
SETA

Cindy Bruckner-Lea
Pacific Northwest National Laboratory

Wayne Bryden
WA Bryden Consulting

Charles Burrus
Metropolitan Transit Authority/New York CityTransit

Patrick Byrne
Department of Health and Human Services

Kathryn Callahan
Department of Homeland Security

Thomas Cebula
Johns Hopkins University

Carol Chapman
Department of Homeland Security

Ari Cohen
Department of Defense

Rita R. Colwell
University of Maryland, College Park
Johns Hopkins University

Tod Companion
Department of Homeland Security

Bernard Courtney
The Tauri Group

David Cullin
FLIR Systems

Ken Damer
Northrop Grumman

Chris Detter
Los Alamos National Laboratory

Donald Eby
Booz Allen Hamilton

Eric Eisenstadt
Private Citizen

Gerald Epstein
Department of Homeland Security

Seth Faith
Battelle Memorial Institute

Michael Farrell
Centers for Disease Control and Prevention

Jeffrey Faszcza
Catalyst Partners

Erik Fiske
Department of Homeland Security

Eric Gard
Lawrence Livermore National Laboratory

Alexander Garza
FirstWatch, Inc.

Jane Getchell
Association of Public Health Laboratories

Alison Graziano
The Tauri Group

David Hanlon
Quanterix

Manzour Hazbon
Private Citizen

Yair Hazi
HWC BioWatch SPO

J. Jerome Holton
The Tauri Group

Karen House
Aberdeen Proving Ground

Scott Hughes
New York City Public Health Laboratory

Anne Hultgren
Department of Homeland Security

Rabih Jabbour
Private Citizen

Diane Jamrog
Lincoln Laboratory
Massachusetts Institute of Technology

Malcolm Johns
Department of Homeland Security

Rudolph Johnson
Centers for Disease Control and Prevention

Franca Jones
Executive Office of the President

Stevan Jovanovich
IntegenX

Robert Kadlec
RPK Consulting

Jordan Kanter
Executive Office of the President

Ivor Knight
Canon U.S. Life Sciences

Charles Kolb
Aerodyne Research, Inc.

Kristin Korte
FLIR

David LaVan
National Institute of Standards and Technology

Matthew Lesho
Luminex Corporation

Beth Maldin Morgenthau
New York City Department of Health and Mental Hygiene

Irene March
The Tauri Group

Raymond Mariella, Jr.
Lawrence Livermore National Laboratory

Mickey McCarter
Homeland Security Today

Atelia McClelland
Hassett Willis and Company

Suzet McKinney
Chicago Department of Public Health

Toby Merlin
Centers for Disease Control and Prevention

Stephen Morse
Columbia University

William Niu
UTC Aerospace System

M. Allen Northrup
Northrup Consulting Group

Joanne Michelle Ocampo
Georgetown University

Michael O'Keefe
The Tauri Group—BioWatch SETA

Ted Olsen
PathSensors

Kristin Omberg
Los Alamos National Laboratory

William O'Neill
U.S. Postal Sevice

Zheng Ouyang
Purdue University

Erica Pan
Alameda County Public Health Department

Andreea Paulopol
U.S. Department of State

David Persse
City of Houston

Sally Phillips
Department of Homeland Security

John Plante
Chicago Transit Authority

Don Prosnitz
Private Citizen

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E

Technology Readiness Levels in the Department of Defense[1]

Uses of Technology Readiness Levels

The primary purpose of using technology readiness levels (TRLs) is to help management in making decisions concerning the development and transitioning of technology. It should be viewed as one of several tools that are needed to manage the progress of research and development activity within an organization.

Among the advantages of TRLs:

- Provide a common understanding of technology status,
- Risk management,
- Used to make decisions concerning technology funding, and
- Used to make decisions concerning transition of technology.

Some of the characteristics of TRLs that limit their utility:

- Readiness does not necessarily fit with appropriateness or technology maturity.
- A mature product may possess a greater or lesser degree of readiness for use in a particular system context than one of lower maturity.
- Numerous factors must be considered, including the relevance of the products' operational environment to the system at hand, as well as the product–system architectural mismatch.

[1] *Technology Readiness Assessment (TRA) Guidance.* U.S. Department of Defense, April 2011.

TABLE E-1 TRL Definitions, Descriptions, and Supporting Information

TRL	Definition	Description	Supporting Information
1	Basic principles observed and reported	Lowest level of technology readiness. Scientific research begins to be translated into applied research and development (R&D). Examples might include paper studies of a technology's basic properties.	Published research that identifies the principles that underlie this technology. References to who, where, when.
2	Technology concept and/or application formulated	Invention begins. Once basic principles are observed, practical applications can be invented. Applications are speculative, and there may be no proof or detailed analysis to support the assumptions. Examples are limited to analytic studies.	Publications or other references that outline the application being considered and that provide analysis to support the concept.
3	Analytical and experimental critical function and/or characteristic proof of concept	Active R&D is initiated. This includes analytical studies and laboratory studies to physically validate the analytical predictions of separate elements of the technology. Examples include components that are not yet integrated or representative.	Results of laboratory tests performed to measure parameters of interest and comparison to analytical predictions for critical subsystems. References to who, where, and when these tests and comparisons were performed.
4	Component and/or breadboard validation in laboratory environment	Basic technological components are integrated to establish that they will work together. This is relatively "low fidelity" compared with the eventual system. Examples include integration of "ad hoc" hardware in the laboratory.	System concepts that have been considered and results from testing laboratory-scale breadboard(s). Reference to who did this work and when. Provide an estimate of how breadboard hardware and test results differ from the expected system goals.

TRL	Definition	Description	Supporting Information
5	Component and/or breadboard validation in relevant environment	Fidelity of breadboard technology increases significantly. The basic technological components are integrated with reasonably realistic supporting elements so they can be tested in a simulated environment. Examples include "high-fidelity" laboratory integration of components.	Results from testing laboratory breadboard system are integrated with other supporting elements in a simulated operational environment. How does the "relevant environment" differ from the expected operational environment? How do the test results compare with expectations? What problems, if any, were encountered? Was the breadboard system refined to more nearly match the expected system goals?
6	System/subsystem model or prototype demonstration in a relevant environment	Representative model or prototype system, which is well beyond that of TRL 5, is tested in a relevant environment. Represents a major step up in a technology's demonstrated readiness. Examples include testing a prototype in a high-fidelity laboratory environment or in a simulated operational environment.	Results from a laboratory testing of a prototype system that is near the desired configuration in terms of performance, weight, and volume. How did the test environment differ from the operational environment? Who performed the tests? How did the test compare with expectations? What problems, if any, were encountered? What are/were the plans, options, or actions to resolve problems before moving to the next level?
7	System prototype demonstration in an operational environment	Prototype near or at planned operational system. Represents a major step up from TRL 6 by requiring demonstration of an actual system prototype in an operational environment (e.g., in an aircraft, in a vehicle, or in space).	Results from testing a prototype system in an operational environment. Who performed the tests? How did the test compare with expectations? What problems, if any, were encountered? What are/were the plans, options, or actions to resolve problems before moving to the next level?

continued

TRL	Definition	Description	Supporting Information
8	Actual system completed and qualified through test and demonstration	Technology has been proven to work in its final form and under expected conditions. In almost all cases, this TRL represents the end of true system development. Examples include developmental test and evaluation (DT&E) of the system in its intended weapon system to determine if it meets design specification.	Results of testing the system in its final configuration under the expected range of environmental conditions in which it will be expected to operate. Assessment of whether it will meet its operational requirements. What problems, if any, were encountered? What are/were the plans, options, or actions to resolve problems before finalizing the design?
9	Actual system proven through successful mission operations	Actual application of the technology in its final form and under mission conditions, such as those encountered in operational test and evaluation (OT&E). Examples include using the system under operational mission conditions.	OT&E reports.

F

The BioWatch Program: What Information Is Needed to Inform Decision Making?

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A white paper prepared for the June 25–26, 2013, workshop on Strategies for Cost-Effective and Flexible Biodetection Systems That Ensure Timely and Accurate Information for Public Health Officials, hosted by the Institute of Medicine's Board on Health Sciences Policy and the National Research Council's Board on Life Sciences. The author is responsible for the content of this article, which does not necessarily represent the views of the Institute of Medicine or the National Research Council.

OVERVIEW OF BIOWATCH AS IT RELATES TO PUBLIC HEALTH OFFICIALS AND LABORATORY SCIENTISTS

The BioWatch Program was put in place in 2003 to monitor the threat of an aerial release of a high-consequence infectious agent within a populous area. It is a first-of-its-kind, bioaerosol monitoring network that spans federal agencies from national security, public health, and environmental protection. The program, overseen by the Department of Homeland Security (DHS), works closely with the Centers for Disease Control and Prevention (CDC), Federal Bureau of Investigation (FBI), Environmental Protection Agency (EPA), and, more recently, with the Department of Defense. While it is managed by the DHS's Office of

Health Affairs, BioWatch itself is maintained and operated at the local level as a unique entity within each jurisdiction. The decision makers within these jurisdictions represent a composite from municipalities, county, state, and regional authorities and may include both public and private entities. No two BioWatch jurisdictional operations, within 30-plus locations, are exactly alike. In general, laboratory testing and much of the program's operational response lie within public health as the lead entity in determining response to an infectious disease event with potential for high-consequence impacts. In response to an intentional release, a strong, collaborative partnership with law enforcement is required to coordinate parallel criminal and public health investigations. Partnerships with FBI and other local law enforcement have been strengthened by the BioWatch Program.

Within the public health community, the BioWatch Program is viewed as one surveillance tool among many; another such tool is, for example, syndromic surveillance, used for detecting the presence of an infectious agent of public health significance. The uniqueness of the BioWatch Program is in its ability to act as an environmental early warning system detecting the presence of a specific agent prior to the appearance of significant human disease. It contributes to the larger surveillance picture. There is a broad array of opinions regarding the need for such an early warning system. A system identifying rare events measured against resources necessary for national public health surveillance systems that identify many public health threats is bound to generate controversy among public health professionals. It is difficult to measure the value of such a system until it has been triggered by the purposeful release of a biothreat agent.

WHAT IS A BAR? WHAT ANALYSIS OR ACTIONS MAY BE TAKEN AS A RESULT OF A BAR?

In oversimplified terms, a BioWatch Actionable Result (BAR) is a laboratory test result indicating that multiple nucleic acid signatures specific to an individual biological agent have been detected in the environment. Prior to determining a BAR, a two-tiered testing process occurs: (1) screening samples for a single signature per agent and (2) confirming any reactives by testing for additional signatures for that agent. None of the results indicate that a viable, or live, organism has been detected. The BAR leads to the public health laboratory director, or a designee, review-

ing laboratory test data and determining whether all internal controls and testing processes are consistent with a "true positive"—that is, the detection of the biological agent. This review includes ensuring that the result is not a laboratory-generated error due to a cross-contamination issue or reagent failure. The laboratory director, or designee, has the option to contact the CDC's Bioterrorism Rapid Response and Advanced Technology Laboratory and DHS's BioWatch Special Program Office (BWSPO) for an outside technical consultation prior to determining a laboratory BAR. This might be done, for example, if there is a known reagent or instrument issue that may impact the result interpretation. Ultimately, the verification or "sign off" of the result by the laboratory director, or designee, assigns professional responsibility in ensuring reliability of the test result.

Once a laboratory BAR has been determined, a number of rapid local and national notifications occur within the jurisdiction, including notification to convene a conference call within 2 hours to discuss the laboratory BAR with the local BioWatch Advisory Committee (BAC), or a BAC subset with key representation. Early in these notifications, the jurisdiction's local FBI weapons of mass destruction coordinator is given a heads-up. Resources may be sought to initiate a request to the BWSPO to seek plume modeling at the national level; some locations use local resources, such as the National Guard's civil support team or other agencies, as a supplementary resource for modeling. In many jurisdictions a local BAC call occurs just prior to the BioWatch national conference call. Some jurisdictions have indicated that the notification time frame is complicated to manage if the BAR is received after work hours or during evening commutes. The BAC chair, typically a public health official, coordinates the jurisdictional BAC conference call. The agenda is quickly covered using a customizable conference call script provided by the BWSPO. The information reviewed includes a concise presentation of the laboratory BAR data, including (1) assurance that the laboratory has reviewed its internal quality testing checklist and (2) a quick primer on how to interpret the data including its limitations, followed by (3) the actual laboratory details, such as number and location of the BioWatch collectors that were positive, the organism detected, the cycle threshold (Ct) value for each collector, and the weather conditions for the preceding 12 to 48 hours.

The BAC chair guides the conference call by requesting supplementary information, such as available intelligence information, presence in the jurisdiction's environment, and any human or veterinary surveillance

data related to the organism detected. At this time a DHS BWSPO request for source reconstruction and plume modeling may already have been initiated, and it is understood that these national laboratory resources require a 6- to 8-hour lead time. Modeling data are used primarily to assist with environmental sampling decisions. A rapid assessment of the perceived threat and the public health risk is performed based on the information in hand. The BAC then decides on a course of action for the following: (1) a decision to initiate environmental sampling; (2) a decision to alter the sampling intervals for the collections (i.e., collecting every 6 to 12 hours versus every 24 hours) or add additional collectors; (3) a decision to initiate a finer focus on syndromic surveillance system results based on the agent detected; (4) identification of the need for federal resources (e.g., support for environmental sampling or testing, initiation of strategic national stockpile resources, if known); (5) implementation of public information and messaging plans; and (6) estimation of the time for the next BAC conference call. The BioWatch national conference call occurs immediately following the local jurisdictional BAC call and begins with a summary by the BAC chair of the current situation, follow-on actions, requests for federal assistance from the various agencies (DHS, CDC, FBI, EPA, or the strategic national stockpile) and a decision regarding the next conference call time.

BENEFITS, LIMITATIONS, AND ISSUES RELATED TO THE GEN-2 DATA GENERATED BY THE CURRENT BIOWATCH PROGRAM

Within the current Biowatch Program, data are generated on a 24-hour cycle by laboratory staff. These data consist of either positive (Ct values) or negative results (not detected, i.e., below the signal threshold) for nucleic acid signatures representing a panel of select biological agents. Millions of results have been generated across the country from a variety of geographic locations using the current Gen-2 biodetection technology: real-time detection polymerase chain reaction (RTD-PCR). A positive aspect of the current RTD-PCR technology is that positive results (Ct values) are semiquantitative.[1] The Ct value is a numerical value indicative of the relative concentration of nucleic acid detected in the

[1]BioWatch Portal: BioWatch laboratory assay.

sample. This information has been useful when considering response actions following a single- versus multiple-collector "hit."

Data generated by the BioWatch Program represent samples collected from a variety of environments (primarily outdoor, but with some limited indoor locations) and spanning the full seasonal variation across the country. The depth and experience with these data have served to establish an important baseline for sensitivity, specificity, reproducibility, and an understanding of the inherent limitations of the current technology in interpreting and acting upon results. One very important aspect of the current Gen-2 system and reagents is that they have not generated laboratory test false-positives but they have detected agents from the panel in the environment, which has operationally complicated the response to a BAR (IOM and NRC, 2011). Additionally, implementation of a robust quality assurance program by the BWSPO[2] in 2010 has resulted in data supporting the reliability of the results and engendering additional confidence. Specifically, qualified laboratory testing staff must demonstrate ongoing individual competency as well as participate in overall laboratory proficiency assessments. Each laboratory is responsible for maintaining all of the components of a comprehensive quality assurance plan (standardized methods, staff training, proficiency tests, stringent record keeping, equipment maintenance, corrective action protocols, etc.) to ensure that high-quality results are reproducibly obtained. The BioWatch Program should be recognized for its significant effort in partnering with each public health laboratory to ensure high-quality results from that BioWatch laboratory.

As indicated earlier, the periodic detection of some of the biothreat agents on the test panel has occurred due to their presence in the natural environment. Several agents in significant enough concentrations in the environment have been detected by the BioWatch network within several geographic locations across the country. While these detections were still "true positives" from the laboratory perspective, they represent laboratory test data that needed to be interpreted in the context of other information. In the first example, one agent (*Brucella* sp.) was detected on multiple occasions in the environment, but the organism was later deemed not to be of sufficient public health concern, and monitoring for this agent was suspended early in 2008.[3] The second example involved the detection of a closely related subspecies of the agent of tularemia

[2]BioWatch Portal: DHS BioWatch Program quality assurance program plan.

[3]BioWatch Portal: DHS memorandum: Recommendation to discontinue monitoring for *Brucella* species from biomonitoring effort.

(*Francisella tularensis* subspecies) not considered to be a human pathogen and therefore not of public health concern. At the time, the laboratory test reagents within the test panel were not specific enough to differentiate between virulent and avirulent subspecies of this organism. Since this event BioWatch has introduced an additional set of laboratory reagents to rapidly differentiate between virulent and avirulent subspecies.[4] These two examples highlight the need for flexibility in the panel of agents detected—to improve upon the performance of a reagent, remove the organism from the panel, or adjust a local threshold—if repeated detections in the natural environment act as a "red herring."

One limitation of the current system has been the length of the turnaround time—the time between when the air sample collection begins and a laboratory test result is reported. Essentially, the existence of the turnaround time translates into the possibility that an aerial release could have occurred any time within a 10- to 36-hour window. For the purposes of accurately pinpointing when an exposure might have occurred, shortening the turnaround time to 4 to 6 hours (or even less if the technology is capable) would provide a significant advantage for public health response. This will be particularly true if the jurisdiction in question expands detection systems to include the transportation sector (e.g., subways, commuter rail, airports). A 4-hour (or less) collection-to-result turnaround time is much more useful in the transportation sector where the exposed cohort rapidly becomes difficult to identify for the purposes of prophylactic treatment or vaccination.

One other limitation to the current technology should be noted. The BioWatch result signifies detection of an agent's nucleic acid signature, but this does not allow the determination of "viability," that is, that the agent is alive and infectious and therefore a risk to human health. The ability to rapidly test agent viability within the sample would be valuable. It is recognized that this may be outside the limits of current technology. Part of the response to each BAR is the collection and testing of environmental samples to determine the presence of viable organism. The culturing of supplementary environmental samples can add an additional 24 to 72 hours in determining the viability of an agent and its full public health significance.

[4]BioWatch Portal: BioWatch Program *Francisella tularensis* guidelines for responding to an outdoor BioWatch Actionable Result.

CONSIDERATIONS RELEVANT TO NEW AUTONOMOUS DETECTOR TECHNOLOGY

The BioWatch Program's initiatives to modernize its bioaerosol detection capability, among other strategic objectives, not only are relevant to the program mission but also are in alignment with a national movement to strengthen the public health infrastructure. The capacity to rapidly identify disease threats or emergent infectious diseases has been increasing, and with it there has been a concomitant need for effective communication and response (Gargano and Hughes, 2013). Recent public health threats and active responses include those to novel Middle East respiratory syndrome coronavirus and avian influenza A (H7N9). There is an urgent need to rapidly improve and modernize laboratory-based technologies that are used to detect and characterize pathogens of public health concern within the national public health laboratory network. To ensure the public health mission of staying abreast of modern disease diagnostics, CDC has initiated a fiscal year 2014 budget request to fund the Advanced Molecular Detection and Response to Infectious Disease Outbreaks initiative.[5] While this effort focuses on next-generation sequencing methods and the application of bioinformatics to public health science and practice, other methodologies, such as mass spectrometry, are rapidly emerging on the clinical diagnostics front (van Belkum et al., 2012) and being adopted within the public health laboratory network. Public health is supportive of the opportunity to adopt new technologies to protect the nation's health.

As a replacement for the Gen-2 system, the following families of biodetection technologies are being considered for an autonomous detection system: nucleic signatures (PCR, microarrays, and other probe-based systems); genomic sequencing; immunoassays and protein signatures; and mass spectroscopy. The goal is to identify a more cost-effective, field-based, autonomous detector with acceptable performance specifications that would result in the ability to improve upon population coverage. Some of the technologies being considered perform best on samples for which the agent has been purified from a complex environment or for which the agent is of sufficient concentration to perform detection and identification. It is likely that a combination of more than one technology

[5]Advanced Molecular Detection and Response to Infectious Disease Outbreaks initiative, http://www.cdc.gov/fmo/topic/Budget%20Information/factsheets/AMD_Factsheet.pdf (accessed August 4, 2013).

will be necessary to achieve collection, concentration, purification, and amplication from a complex matrix followed by detection to meet the specifications required of the new system (Sapsford et al., 2008). Some of the new technologies may afford the ability to "fingerprint" the agent detected by providing strain-level discrimination. While this information is important to confirm a source, it may not add immediate value to public health response decisions unless the information improves upon the ability to discriminate between closely related species. Examples include differentiation between virulent and avirulent *Francisella tularensis* subspecies or the resolution of closely related species such as *Burkholderia mallei* and *Burkholderia pseudomallei*. This level of specificity would be useful in weighing disease risk and the need for prophylaxis.

While public health is receptive to adopting a new technology, many of same considerations and responsibilities for interpreting a Gen-2 BAR will still apply for the new autonomous detector technology. A simple "red light/green light" result does not provide sufficient data to a public health laboratory official to make a professional determination regarding the quality of the result. This is especially true of adopting a new technology for which the laboratory test would be performed at a remote location. There is no precedent for this in the public health laboratory realm with any technology. The closest is the U.S. Postal Service Biological Detection System (BDS) for which a "positive" result is not confirmed until the BDS cartridge is brought to the public health laboratory and confirmed using CDC's Laboratory Response Network (LRN) reagents. To call a BAR on data generated by an instrument in the field, there must be access to data to review the following: instrument quality performance indicators (these will vary depending on the technology), positive and negative controls, threshold settings, and the actual test value (qualitative or quantitative). Other parameters are critical to test interpretation: preknowledge of the autonomous detector's sensitivity, specificity, reproducibility (machine-to-machine, day-to-day); robustness (environment-to-environment, season-to-season); and limitations (adverse impacts of environmental contaminants, such as pollen or brake dust). It will be important for any new technology to be benchmarked against the current technology. A specific protocol is in place describing the mechanism for introducing a new platform or method to the BioWatch Program.[6] It should be assumed that the new technology must be at least equal to the current technology (Gen-2 RTD-PCR) in sensitiv-

[6]BioWatch Portal: New method, assay, and platform acceptance testing guidance.

ity, specificity, reproducibility, and instrument reliability before it is adopted. As with Gen-2, data generated by the new autonomous detection system will still need to be interpreted within the context of other information.

Additional operational and technical considerations include the following:

- Reducing the detection turnaround time to 4 to 6 hours means that the testing frequency will be approximately 5 to 7 times more than the current Gen-2 technology. The new technology will have to perform proportionally better to not suffer the consequences of an increase in "actionable" results that turn out to be false-positives or instrument errors.
- Being able to have some flexibility in the agent panel by allowing addition of new threat agents or removal of others, should the need arise, would be useful.
- Being able to adjust the duration of the collection and testing window in response to a BAR should be considered.
- Ensuring secure and reliable electronic results from each instrument is of concern. Information security and internet stability will require redundancy in the system. Preventive measures to prevent spoofing a sensor or modification of transmitted data must be ensured.
- Replicating key aspects of the current laboratory-based quality assurance program will be necessary to ensure high-quality results from each instrument and the system as a whole.
- Other factors that will affect the placement of an autonomous detector include instrument footprint, weight, and operating noise levels.
- Colocation of environmental monitors for such measures as temperature and humidity would be useful in evaluating the impact on instrument performance from—as well as providing data on—the environmental conditions at the time of an instrument BAR.
- Saving a portion of the test sample to verify by other test methods would be highly desirable.
- Provision for the option for some jurisdictions to perform basic autonomous detection reagent loading and quality checks is desirable and would facilitate a familiarity with the technology and its inherent limitations. This "boots on the ground" feature would

provide valuable insight into technical problems and their solutions and would ensure a greater confidence in interpreting data from the autonomous detector by the responsible laboratory director, or designee.

In conclusion, the evaluation and adoption of a new technology to improve and automate the BioWatch network will be supported if the technology's performance characteristics are equal to or better than those of the existing Gen-2 laboratory-based operation. The public health laboratory must be provided with sufficient data to be familiar with the instrument's performance, including its limitations, in order to instill trust and confidence in the quality of the data. This is critical to ensure technical competence and expertise in interpreting the results of an autonomous detector BAR for public health officials and other key stakeholders involved in response actions.

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G

Nucleic-Acid Signatures at Three Levels of Readiness for BioWatch

R. Mariella, Jr., Ph.D.

A white paper prepared for the June 25–26, 2013, workshop on Strategies for Cost-Effective and Flexible Biodetection Systems That Ensure Timely and Accurate Information for Public Health Officials, hosted by the Institute of Medicine's Board on Health Sciences Policy and the National Research Council's Board on Life Sciences. The author is responsible for the content of this article, which does not necessarily represent the views of the Institute of Medicine or the National Research Council.

The paper by Raymond P. Mariella, Jr., will discuss nucleic-acid signatures (polymerase chain reaction [PCR], microarrays, and other probe-based systems) at three levels of readiness for BioWatch:

- Tier 1: fully automated biodetection system, capable of 24/7/365 unattended outdoor and indoor operation, that will be at a technology readiness level of technology readiness level (TRL) 6-plus by 2016.
- Tier 2: similar requirements but will not reach a TRL 6-plus level until sometime between 2016 and 2020.
- Tier 3: technologies that have the potential of meeting or exceeding the BioWatch requirements, but a fully automated, TRL 6-plus system would take us beyond the 2020 time frame. For these technologies, describe the current critical paths ("long poles") in meeting the BioWatch requirements and how they might be addressed.

DISCLAIMER

This paper, focusing on nucleic acid–based detection of bioaerosol agents, must be only a subset of a larger plan. This paper does not address foodborne, waterborne, vectorborne, or human-to-human transmission of infectious agents.

AUTHOR'S STRONG RECOMMENDATION FOR BOTH PLANNING AND IMPLEMENTATION

Because the committee is examining what would be entailed to provide an autonomous system that performed BioWatch assays and provided the Laboratory Response Network (LRN) of the Centers for Disease Control and Prevention (CDC) with the same amount of nucleic-acid sequence information, along with the same reliability and accuracy as the validated laboratory assays, in the author's opinion the CDC/LRN and any others who will rely upon such information to make calls of detection of an agent need to be involved in each step in the planning and development of such an autonomous system.

CAVEAT: FULL, END-TO-END SYSTEM INTEGRATION IS DIFFICULT!

The author's general premise is that the integration of disparate components into an automated bioagent detection and identification system that attempts to perform the same functions as trained laboratory personnel is extraordinarily difficult. Numerous high-quality commercial off-the-shelf instruments exist that perform the functions of the individual steps, yet field-tested and proven integrated systems are few. It is a particular challenge in any automated fluidic system to make 100 percent reliable fluidic interconnections along with the necessary methods to transport, meter, and process samples and reagents so that the laboratory-proven assays that normally are carried out by trained personnel with pipettes, filters, and centrifuges perform just as well in the automated system. Challenges to system integration can arise from something as basic as an output volume of one stage being incompatible with the next stage's input volume or from differing buffer concentrations between one stage and the next or from a "short spin" on the centrifuge being needed

in between, and so on. One extremely important hurdle for system integration is that a manufacturer may refuse to provide what seems to be a minor modification to a selected component that is needed to permit integration, and, if a third party performed the needed modification, the original manufacturer may refuse to service the modified component and declare that the modification voided the warranty. This is particularly likely when the desired component might be projected to have total annual sales of a few dozen or less. Thus, a stand-alone instrument may work very well in an LRN or BioWatch laboratory and, yet, effectively not be available for system integration.

INTRODUCTORY THOUGHTS AND OVERVIEW

In Figure G-1, items 2 through 4 and 6 through 11 are either incorporated or could easily be incorporated in systems that could be deployed by 2016. Item 1, precollection particle characterizer, could probably be incorporated as part of a next-generation system by 2016, but certainly by 2020, if the cost-benefit analysis favored its incorporation. Item 5, selective capture/enrichment of pathogens via surface molecular recognition is listed, although extensive research and development would be required to make this a reality (Pratt et al., 2011). Item 8 is also somewhat of a catchall, covering simple chemistries, such as the Boom capture/release of nucleic acids, but may also include emerging techniques, including even capture, isolation, and release of nucleic-acid regions of interest on either fixed or liquid microarrays (DuBose et al., 2013). Item 12 would likely require its own multiplex target amplification, prior to hybridization, and incorporating such a process, along with the logistics of replacement of single-use microarrays or the reconditioning of a microarray, along with automated readout of hybridization patterns, seems destined for deployment beyond 2020. Item 13 would perform a very desirable function, but attempting an automated metagenomic analysis of a sample seems like a very "long pole" in the tent. Preceding sequencing with multiplex target amplification may make this automatable by 2020.

PERFORMANCE CONSIDERATIONS

Premise

A release of agent can dissipate and yet remain at physiologically relevant concentrations, for large areas, putting the public at risk, so the aerosol sampler needs to bring in as many liters per minute as is practical.

Considerations

Without regard to any specific autonomous system or placement of a multitude of such systems throughout an urban setting, let us simply consider what might happen to persons who were exposed for 30 minutes to a release that contained *N* viable bacteria/m^3.

From Chapter 6 of the Environmental Protection Agency (EPA) *Exposure Factors Handbook* (Moya et al., 2011), adult humans respire, on a 24-hour average, roughly 10 liters/minute, so 30 minutes = 300 liters breathed. Because 1,000 L= $1m^3$, "average" breathing would pull in 0.3 N viable bacteria over 30 minutes. Depending upon the details of the aerosol, it could happen that effectively every viable bacterium breathed in could be retained within the lungs.

The infectious dose of some bacteria and viruses has been measured to be essentially a single viable bacterium or particle with infectious virions (Jones et al., 2005). Regarding viruses, according to Kamps (2006):

> Within nasal secretions, millions of virus particles per ml are shed, so that a 0.1–µ1 aerosol particle contains more than 100 virus particles. A single HID (human infectious) dose of influenza virus might be between 100 and 1,000 particles. (p. 91)

Although respiration rates among the very young or the elderly may be lower, the infectious dose for some agents may be lower as well. According to Ingles by et al. (2002):

> Recently published extrapolations from primate data suggest that as few as 1 to 3 spores may be sufficient to cause infection (Peters and Hartley, 2002). The dose of spores that caused infection in any of the 11 patients with inhalational anthrax in 2001 could not be estimated although the 2 cases of fatal inhalational anthrax in New York City and Connecticut provoked speculation that the fatal dose, at least in some individuals, may be quite low. (p. 2239)

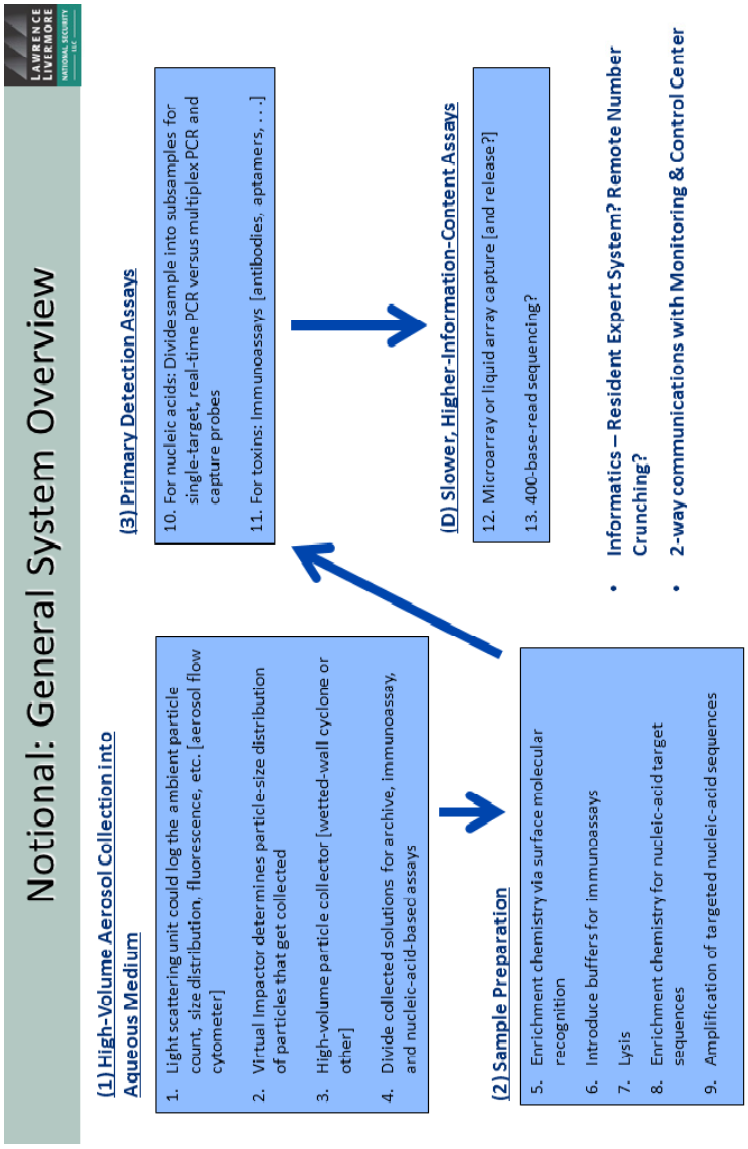

FIGURE G-1 Block diagram of components of a notional next-generation system, including components and functions that may be incorporated in a staged manner.

Thus, if *N* for a particular agent remained above $10/m^3$, for example, then a 30-minute exposure could lead to infection of persons in that vicinity if the infectious dose was very low. The location of an autonomous system may be far enough away that the aerosol concentration of potentially pathogenic bacteria or virus may have dropped to a much lower level when the release reached the detection system; indeed, this appendix author argues that the farther apart one wishes to locate such autonomous systems, the higher each system's volumetric sampling and sensitivity need to be to avoid missing an attack.

Regarding background bioaerosols, Jensen (2007) reported:

> Time-resolved concentration profiles of bacteria and spores showed that spore concentrations of 11,000 per cubic meter occurred and that the concentration curve contained many "bumps" resembling an aerosol cloud passage as well as many short duration spikes. (p. 9)

Jensen added that "there were approximately 50 total airborne bacteria for every culturable bacterium" (p. 9), which is of relevance for assays, such as PCR and most other nucleic acid–sequence-based assays, that do not distinguish between "live" and "dead" bacteria.

All of this is to say that it is not hard to envision a situation in which a potentially pathogenic bacteria or virus could be present at physiologically relevant concentrations, while remaining well below the background concentration of physically similar, nonpathogenic aerosolized bacteria and viruses.

NUCLEIC-ACID SIGNATURES

What comprises nucleic-acid signatures? Should an autonomous system include genomic, plasmid, and even rRNA in bacteria by 2020? Concerning informatics, what level of sequence ID is required to call a positive? How well conserved are "consensus sequences"? How much deviation is acceptable? (This is an especially tricky issue for single-stranded DNA and RNA viruses.) Experimentally, what level of sequence mismatch or incorrect base calling is acceptable?

How do signatures need to change as strains evolve or as new species and subspecies are discovered (Jackson et al., 2012; Signarovitz et al., 2012; Zeytun et al., 2012), both within the species of the pathogen and with its near neighbors?

The author expects that such discussions of nucleic-acid signatures and their evolution over time will proceed within the workshop panels "BioWatch—Information for Decision Making" and "State of the Art on Genomic Sequencing." However, this author wishes to convey, anecdotally, that it is his understanding that for at least one species of pathogenic virus there are no conserved sequences longer than 14 bases, making a single-target assay problematic for that agent. Moreover, for single-stranded DNA and RNA viruses, concepts such as "quasispecies" are often needed and appropriate (Lauring and Andino, 2010; Volz et al., 2013).

ASSAYS

LRN and BioWatch laboratory assays today are certainly performed with commercial instruments, but due to the considerations at the beginning of this report on the challenges of system integration, we will not specifically compare various commercial, stand-alone components or instruments. However, the author hopes what follows will be discussion provoking.

Digital Sample Processing and Digital PCR

Given the rigor and time-consuming nature of creating fully tested and validated assays within the guidance of public health actionable assays,[1] digital PCR is likely not fieldable by 2016, but could possibly be TRL 6 by 2020 (Vogelstein and Kinzler, 1999).

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[1] See "Framework for a Biothreat Field Response Mission Capability," developed by an interagency working group convened to develop guidance to first responders for the biological assessment of suspicious powders.

Public health actionable assays (PHAAs) are laboratory-based assays that are used to support public health decisions and which have been qualified according to consensus performance standards developed by a recognized and representative body from the stakeholder community. PHAAs are developed and utilized to support public health actions involving the potential exposure of an individual or, more commonly, groups of individuals to biothreat materials such as *Ba* spores. PHAAs have high specificity, high sensitivity, and are highly robust to provide critical information on agent-specific confirmation and further characterization to support public health decisions such as initiating a national or local health alert warning, initiating a public health investigation, conducting risk assessments to support post-exposure prophylaxis distribution, and initiating public health risk communications. These assays are intended to be employed in well-established controlled laboratory environments,

Gevensleben and colleagues (2013) detected and quantified circulating free plasma DNA with droplet-digital PCR (ddPCR); Strain et al. (2013) did HIV quantification as did Henrich et al. (2012). Kelley et al. (2013) reported sensitive quantification of methicillin-resistant *Staphylococcus aureus* using digital PCR. Roberts and colleagues (2013) described a digital PCR diagnostic assay for ocular *Chlamydia trachomatis*; unlike other nucleic-acid amplification tests, it "requires no external or internal calibration yet delivers a highly accurate estimation of target load."

Speaking about quantification, Morisset and colleagues (2013) wrote:

> The excellent performance of the tested parameters enables the quantification of samples from different matrices, using DNA extracted with common methods without up-front DNA quantity estimation. The limits of quantification, trueness, and repeatability of the duplex assay comply with international recommendations.

They also noted that "ddPCR [droplet digital polymerase chain reaction] running costs are lower than those of standard qPCR [quantitative polymerase chain reaction] technology, given the superior throughput."

There are numerous other publications that used a variety of platforms (Ottesen et al., 2006; Shen et al., 2011; Straub et al., 2013; Tadmor et al., 2011; Whale et al., 2012). Exactly how a system integrator would implement digital PCR, even by 2020, is an open question, but worth considering.

An Aside on Limits of Detection[2]

Apropos of the previous discussion of "digital PCR," but without diving into what is famously known as "receiver operating characteristic" curves, let us briefly review what is meant by a "false-negative." It has been demonstrated, for almost as long as PCR has been used, that an optimized PCR assay can perform down to the Poisson limit of detection

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such as an LRN reference laboratory using an established ConOps and where professional training and user proficiency certification are established.

[2] This author understands that a careful study was made of BioWatch assays, implemented under both single-plex and multiplex formats, but as of this writing he has not been able to read any resulting report. Such a study would bear upon the discussion about limits of detection and Poisson statistics in the preceding section.

(Sykes et al., 1992). That is, if the PCR reaction solution has no inhibitors but does have the appropriate primers, master mix, and only a single copy of the target DNA, a positive detection signal can be observed (Sykes et al., 1992). Without referring to any particular autonomous instrument, if it collects 1 m^3/min and has a 10 percent overall processing throughput from collection to PCR reaction, including archiving and dividing some of the collected sample, then if a particular bacterium enters the collector as an aerosol with concentration of 10/m^3 and the system collects air for 1 minute, then on average the PCR reaction will have one bacteria target in its reaction volume. However, assuming that Poisson statistics apply, 1/e = 37 percent of the time there will be no target in the PCR reaction, and one can, therefore, expect to "miss" the detection of this aerosolized bacteria at this aerosol concentration 37 percent of the time. By contrast, if there were, say, three separate PCR chambers, each with 10 percent overall throughput from collector to it, each with a different sequence signature from the bacterium, then the bacterium would miss all three reactions only $1/e^3$ = 5 percent of the time. If the manufacturer claimed a limit of detection *with* all three signatures positive for this bacterium of 10/m^3 in 1 minute, then the system would fail to give this positive 37 percent of the time. If only one or two signatures came up positive, the result could be termed "indeterminate," for example, with its own particular concept of operation.

Also note that if a multiplex assay with three sequence signatures per target organism also obeyed Poisson statistics and was incorporated in a similar autonomous system that had 30 percent throughput from collector to reaction chamber, because of having less division of the collected sample, it would produce a false-negative 5 percent of the time for this concentration of aerosol.

Topics for Probe-Based Assays

- What are the accuracy, time to perform, cost . . .?
- Sensitivity, specificity: Should require positive call for all or only some physiologically relevant concentrations? No false-positives from nonvirulent subspecies.
- Time to perform assay (Maltezos et al., 2010): "Using *Thermococcus kodakaraensis* polymerase . . . we obtained an accurate, 35-cycle amplification of an 85-base pair fragment of *E. coli O157:H7 Shiga toxin gene* in as little as 94.1 s." It seems unlikely that the time to perform the polymerase-based amplification will

be the limiting factor on processing time, even with slower thermal cyclers.

- Difficulty to perform manually and/or automatically.
- What level of sample preparation is required?
- Ability to transfer informatics, reagents to other locations.
- Cost of startup for each detection system, consumables, instrument maintenance.
- Ability to include positive and negative controls.
- Expandability: multiplex versus single-plex.
- What protocol and procedures are required for validation (PHAA)? Time, number of assays to run, experimental accuracy, etc.

Should there be at least two simultaneous or sequential hybridization events required per signature region, with at least three signatures required per target bacterium or virus?

This author, in contacting numerous practitioners, has found divergent positions on the topic of which assays to incorporate into autonomous detection systems. A number of skilled laboratorians endorse the BioPlex® assay, based on multiplex PCR amplification and subsequent hybridization to a liquid array of bead types. A different group of skilled laboratorians endorses real-time PCR with Taqman® or Molecular Beacon® probes. The BioPlex® assay was fielded for the Department of Homeland Security (DHS) in the Northrop Grumman autonomous pathogen detector system, with seemingly no false-positives or false-negatives. The real-time PCR was tested in the Hamilton Sundstrand M-BAND in realistic settings with good results. Given the rushed nature of the preparation of this report and the relative lack of overwhelming data, no specific recommendation is made in this report, other than to recommend detailing the time and trouble, within the PHAA guidelines (see footnote 2), to produce and validate each approach, both as individual signatures evolve and as the number of target bacteria and viruses increases. Will positive and negative controls be required for each reaction volume?

Role of Viability/Infectivity Assays?

Part of a long-term system design could include local viability assays, triggered by initial positive identification with nucleic-acid signature. Rapid viability assays have been developed that can even be applied to slow-germinating *Bacillus* endospores (Kane et al., 2006; Letant et al.,

2010, 2011), but using them would require profound changes in automation, because actual culture is required. For some endospores, an accelerated method can still require 12 to 24 hours for accurate results (Letant et al., 2011).

Incorporation would require an analysis of the trade-offs between dispatching a courier to retrieve a positive sample for viability and other testing at a staffed laboratory versus attempting to incorporate such an assay in a stand-alone system or some combination of both.

Sequencing by Hybridization to Planar Array

The author sees sequencing by hybridization (Drmanac et al., 1989; Palacios et al., 2007; Thissen et al., 2010) as having major obstacles to incorporation into a stand-alone, autonomous system, even by 2020. Topics such as probe length and the stringency of hybridization specificity, informatics and pattern-recognition imaging equipment and software, and trade-offs between copy number, size of array, and time to equilibrate can probably be addressed by 2020, but can sample preparation be automated, and what sort of consumables supply and robotic handling of chips for performing assays would be needed? Would the system reuse chips or sequentially use minichips on a larger chip with complicated fluidics? What accuracy results from hybridizing with a complex environmental mixture? To what extent could selective multiplex amplification of regions of interest knock down confounding sequences and nonspecific binding?

To this author, the "long poles" for this application are establishing and automating the sample preparation that is needed prior to hybridization as well as robotic chip handling and the necessary fluidics for introducing new chips and enabling equilibrated hybridizations. The overall cost versus performance would also need to be considered.

Direct Sequencing of Probe Regions

Direct sequencing of probe regions can be done via pyrosequencing, single-molecule sequencing, etc. Although next-generation sequencing continues to increase the power and affordability of sequencing a human's genome, how well do these increases enhance rapid, autonomous sequencing of pathogenic bacteria and viruses from complex environmental samples?

There are a variety of issues concerning sample preparation for direct sequencing. Starting from a raw aerosol sample—such as that captured in a wetted-wall particle collector, for example—ideally a fully automated system should be able to perform the following tasks:

- Possible selective capture of virulent bacteria and viruses (enrichment of pathogens out of a mixture of innocuous microbes and human detritus) using a surface-recognition capture, possibly with derivatized magnetic beads;
- Spore and cell lysis;
- Extraction and purification of nucleic acids;
- Possible enrichment and capture of nucleic-acid sequences that are signatures of virulence in human pathogens;
- Whatever amplification is necessary (perhaps priming off of the virulence regions);
- Fragmentation and size selection, as needed;
- Addition of bar codes, as needed;
- Ligation, as needed;
- Adjustment of buffers, washing, and separation, as needed for every step to produce the necessary sequencing library;
- Sequencing with each read being ≈ 400 bases;
- Maybe quantification of starting copy number; and
- Informatics analysis of sequence, including "identification" of strain and substrain variants (which is particularly tricky for ss-DNA or RNA viruses) resulting in actionable output.

INSTRUMENTS, PLATFORMS, AND PROBLEMS

There are various issues related to instruments and platforms. Concerning polymerase-based assays that use thermal cyclers, for example, if disposable, single-use tubes or wells are used, what burden of consumables is incurred? If flow-through instruments are used, what difficulties will arise in cleaning out all prior material? There are also issues related to the lifetime and aging of the flow-through, thermal-cycling chambers and tubing, such as what happens with repeated exposure to bleach.

If fixed surface is used, such as a microarray, how does one integrate the flow system and hybridization chamber—swish the sample back and forth or use diffusion, only? What about the time for equilibration/signal versus the starting copy number and the concentration of possibly inter-

fering sequences? There are also questions about the size and cost of each array.

What sample preparation is needed, including either nonspecific or specific (i.e., a virulence region) and multiplexed amplification (similar to AmpliSeq®, but targeted to "regions of interest").

Concerning the leak-free insertion of a new array for the next sample, what complexity and reliability of robotics would be required? How about a large microarray with many subarrays in one large fluidic manifold, where each microarray is operated individually?

What pattern-recognition software exists or needs to be created? What will be need to distinguish multiple targets simultaneously? What about reproducibility and quantification?

Would the DHS application require a custom sequencing platform? Existing systems that slowly provide 10^8 or 10^9 reads do not seem to be a good match to the DHS workspace. Also, the carryover of reads from prior sequencing runs could prevent simple adoption of an existing sequencing platform.

What choices are there for robotic or microfluidic platforms to perform sample handling and sample preparation for a customized sequencing instrument? One is IntegenX, but there are others.

What software is needed to reduce or eliminate the local need for experienced informatics personnel to interpret sequence data, including such things as natural sequence variability and also instrumental miscalls? If resident, expert software is feasible, what is its time frame to reach TRL 6? It is this author's understanding that multiple groups are already working on such software, so a system with custom sequencing and resident, expert software could be available in the 2016–2020 time frame.

Performance of both microarray platforms and sequencing platforms would likely benefit significantly from targeted multiplex polymerase amplification of regions of interest. There would be less confounding data and chemistry in the readout step, and the pattern-recognition or base-calling software would have an easier job.

FIELD-TESTED AUTONOMOUS SYSTEMS

There are a number of mature candidates for field-tested autonomous systems. At TRL 9 is the Northrop Grumman autonomous pathogen detector system, which has performed autonomous operations using multi-

plexed PCR assays for the DHS for some period with no false-positives and no false-negatives. The general operation has been described by Regan et al. (2008) (see Figure G-2).

At TRL 8 is the Hamilton Sundstrand M-BAND PositiveID, which has successfully detected and identified blind samples provided by the DHS, as described by Sanchez et al. (2011) (see Figure G-3).

Both the Northrop Grumman system, which collects 1.7 m^3/min, and the Hamilton Sundstrand system, which collects 0.4 m^3/min, are well beyond the TRL 6 point for field deployment by 2016, assuming no fundamental changes in their assays. The Northrop Grumman system appears to have an easier path forward to expansion of the number of assays (the liquid array approach has a built-in capability to handle up to 100 target sequences) and runs internal positive and negative controls for every polymerase-amplification reaction.

Because of the demand for inexpensive sequencing of individual human genomes, some relevant science and technology continue to advance—not an integrated, fully automated stand-alone system, but good long-term prospects. One example is IntegenX, an automated sample preparation for sequencing (see Figure G-4).

FIGURE G-2 Autonomous pathogen detector system.

FIGURE G-3 M-BAND.

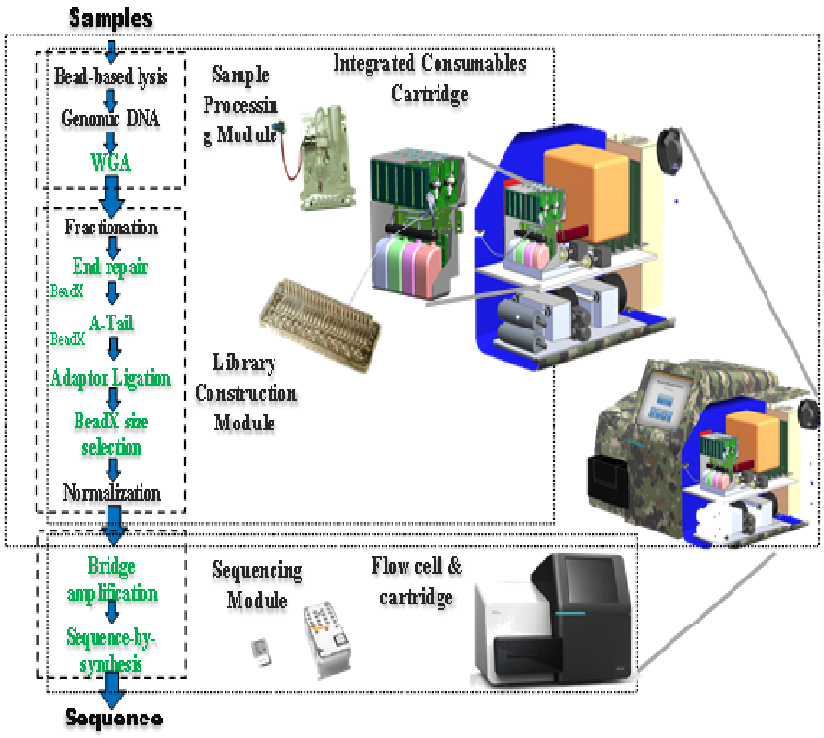

FIGURE G-4 IntegenX.

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H

State of the Art for Autonomous Detection Systems Using Immunoassays and Protein Signatures

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A white paper prepared for the June 25–26, 2013, workshop on Strategies for Cost-Effective and Flexible Biodetection Systems That Ensure Timely and Accurate Information for Public Health Officials, hosted by the Institute of Medicine's Board on Health Sciences Policy and the National Research Council's Board on Life Sciences. The author is responsible for the content of this article, which does not necessarily represent the views of the Institute of Medicine or the National Research Council.

INTRODUCTION

Antibody-based strategies dominated the biological agent detection landscape during the 1980s and well into the 1990s. Antibodies have been used by the U.S. military for the detection of biological warfare agents since the first Gulf War. The ability of immune systems to identify and synthesize immunoglobulins against non-self-structural elements, primarily proteins, with excellent sensitivity and specificity is the backbone of antibody-based detection and identification approaches.

The introduction of nucleic acid–based detection and identification technologies, primarily nucleic-acid amplification technologies, in the later 1980s presented an ability to look at the genetic information in addition to the structural elements of pathogenic organisms. While systems such as BioWatch have focused almost exclusively on nucleic-acid amplification technologies, the continued development of antibody- and

protein-based recognition systems warrants a fresh look at the state of maturity of structural recognition approaches to biological identification. This paper highlights some of the more mature and promising technologies that could be adapted by the BioWatch Program for the identification of harmful biological agents.

All of the systems discussed in this paper can meet or exceed the detection requirements established for the next generation of BioWatch. The advantage of antibody-based and other protein-based technologies is speed. Many of these systems provide answers in 3 to 10 minutes and can operate in a continuous fashion at reasonably low cost with minimal infrastructure support. Some of the systems can reach sensitivities approaching that of nucleic-acid amplification.

STATEMENT OF THE PROBLEM

The Department of Homeland Security (DHS) Office of Health Affairs is researching the potential to employ a fully autonomous networked biodetection capability that will be deployed, operated, and sustained, both indoor and outdoor, in selected BioWatch jurisdictions throughout the United States to continuously monitor the air for agents of biological concern. This requirement supports the DHS plans to increase the capability of the BioWatch system by augmenting and ultimately replacing the current collection and biodetection capability with an autonomous biodetection capability that will improve timeliness, time resolution, population coverage, and cost-effectiveness while enabling the program to stay within its fiscal constraints.

The autonomous detection capability must be able to (1) rapidly process and accurately analyze aerosol samples with a high level of confidence; (2) automate and integrate the major system functions into the detector, including aerosol sample collection, preparation, analysis, and analytical results reporting; (3) operate in its intended indoor and outdoor environments; and (4) disseminate and archive analysis results and system operational data via the C3 network, known as the BioWatch Gen-3 Operations Support Service. The requirement is for autonomous biodetectors, for both indoor and outdoor use, that continuously monitor the air for agents of biological concern (24 hours per day, 365 days per year) and all necessary information technology, equipment, consumables, and technical support for the nationwide deployment, operations, and maintenance of this autonomous biodetection capability.

Appropriateness to BioWatch Problem

The use of antibodies, or immunoglobins, as a structural recognition element in a biological detection architecture takes advantage of one of the body's premier defense systems. Antibodies represent the body's ability to discriminate between self and non-self. The standard IgG immunoglobin is a Y-shaped molecule with the short arms representing identical binding elements and a base that can be anchored to a variety of surfaces. The binding region recognizes a small region, generally three to five amino acids in size, which is unique from the host that generated the antibody as part of an immune response. A single immune cell makes a single antibody structure generating "monoclonal" antibodies. The collection of individual antibodies from blood of a host represents a multitude of these individual antibodies and is termed polyclonal, each individual antibody targeting a unique structure.

The antibodies are responsible for binding foreign entities to target them for destruction and removal from the body. The binding is specific and sensitive. Normal ranges of disassociation constants (indicating tightness of fit) range from 10^{-9} to 10^{-11}. These values are important because they are central in determining the sensitivity and specificity of a detection system utilizing the antibody as a detection element.

Biological identification systems rely on the collection and detection of recognizable entities that are unique to the harmful biological agent and not found independent of it. The BioWatch Program is designed to protect against a catastrophic event caused by bacteria or viruses. Toxins are also a concern because they are potent at very low levels and represent a challenge to nucleic acid–based detection systems. The argument that nucleic acids may contaminate the preparation has merit, but antibody-based systems are more effective and consistent than nucleic acid–based systems for the detection and identification of toxins. In addition, there are some antibody-based systems that provide detection and discrimination of microorganisms at levels that support the autonomous detection requirements established for BioWatch Gen-3.

Sample Collection and Processing

Sample collection and processing are the ultimate driver for sensitivity. The fundamental objective for biological sample collection and preparation is to extract the target signatures from the environment into the volume required for one or more specific assays. A front-end air-

sampling device typically removes particles of defined size and mass into a collection matrix, generally an aqueous liquid. This liquid undergoes extraction(s) of various types to separate the biological signature(s) of interest from the clutter in the sample. As tests are run on smaller volumes, the concentration of target must increase, given a fixed level of detection. If a given test has a sensitivity of 100 signatures, then the concentration (signatures/ml) is indicated in Table H-1. The signature may be a genetic sequence, an epitope for antibody binding, or a mass spectrometer target.

If the copy number required for detection decreases by a factor of 10, then all of the concentration numbers also decrease by that factor. This simple table highlights the requirement for efficient collection and processing for analysis of biological targets.

Signature copy number from a single organism can be larger than the actual copy number of the organism in multiple ways. Most of the antibodies are directed against antigenic determinants that are present on the surface of the cell. Generally there are hundreds to thousands of copies of these structures on a single bacterium or virus. If the target organisms are sonicated to release the soluble contents, hundreds to thousands of membrane fragments can be released from a single organism (each behaving as a separate entity), providing many more independent binding opportunities and dramatically increasing the sensitivity of the system. In this way antibody detection and identification can compete with sensitivities achieved by nucleic-acid amplification technologies.

TABLE H-1 Required Concentrations

Copy Number Required	Volume	Copy Number/ml Required
100	1.0 ml	100
100	1.0 ul	100,000
100	1.0 pl	100,000,000
100	1.0 fl	100,000,000,000

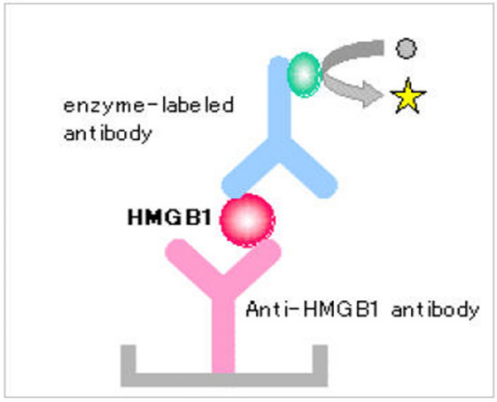
NOTE: fl = femtoliter; ml = milliliter; pl = picoliter; ul = microliter.

Antibody–Antigen Interaction

The vast majority of detection technologies that employ antibodies as structural recognition elements involve initial immobilization of a capture antibody on a solid substrate. This substrate can be a membrane, as in traditional lateral flow assays. Alternatively, an optically transparentmatrix can be used for evanescent wave utilization of suspended substrates, such as a bead used in magnetic separations or optical laser discrimination.

Figure H-1 illustrates a basic approach, referred to as a sandwich assay, for the utilization of antibodies. The primary, or capture, antibody binds the target in such a way that the target is then associated with the capture substrate.

Both antigen and reporter antibody binding events are critical to the fidelity of the detection system. Nonspecific binding is caused by a label, or reporter molecule, being localized with the capture antibody, and it creates noise in the system. It is generally accepted that a specific signal must be at least three times the intensity of the background to be a specific binding event.



The HMGB1 Elisa Kit is a 2-step sandwich ELISA.

FIGURE H-1 Visual representation of the sandwich-style assay in which the capture antibody (pink) is immobilized to a substrate. The red HMGB1 represents the target that binds the capture antibody and is subsequently bound by the blue reporter antibody.
NOTE: ELISA = enzyme-linked immunosorbent assay; HMGB1 = high-mobility group box 1 protein.
SOURCE: http://dc184.4shared.com/doc/goS0-D6B/preview.html.

Reporter Molecules

The reporter molecule is a readable signal that represents what is measured by the system and is intended to be a function of amount of target present in the sample. A wide variety of reporter systems are used in antibody-binding detection methods and represent a significant variable in the sensitivity of various systems. The types of reporter systems can be divided into three broad categories. The first is optical reflectance, such as the colloidal label on secondary antibodies utilized in most lateral flow devices. The second is fluorescence whereby a reporter molecule is excited at one wavelength and then emits light at a different wavelength. The spectral and temporal excitation and emission differences allow various methods of collection to enhance the signal-to-noise difference. The third method is an enzymatic reaction whereby a nonvisible substrate is converted into a detectable product. The enzymatic products vary with different systems.

PLATFORMS

This section presents a variety of different platforms, all of which have been incorporated into a product that can reach TRL 6-plus between 2016 and 2020.

Luminex

The Luminex platform can accommodate both antibody-based and nucleic acid–based biological detection schemes. It is presented first because it is relatively mature and is incorporated into the current BioWatch Program. The Gen-2 BioWatch Program has used the Luminex LX-200 platform to run a polymerase chain reaction (PCR) nucleic-acid amplification–based test. The LX-200 is also integrated in an autonomous PCR-based Gen-3 prototype that has undergone substantial field testing. A modification of the Gen-3 prototype includes the newer MagPix instrument that employs magnetic beads and in which a robust charge-coupled device (CCD) camera replaces the flow cytometer in the LX-200. For antibody-based detection, Luminex uses a bead-based platform in which the capture antibodies are covalently linked to color-coated beads. The BioWatch Program uses the LX-200 system, which incorporates a laser and a complex fluidics system. For future systems

the newer MagPix instrument is recommended as it eliminates the flow cytometer laser and complex fluidics. Figure H-2 illustrates the Luminex open architecture platform that allows for the incorporation of new assays into an existing menu of tests.

Figure H-3 illustrates the detection mechanism for the MagPix system, which uses LEDs for bead optical interrogation and immobilizes the beads with a magnet to allow for increased image capture time, thereby improving sensitivity.

Figure H-4 shows the Magpix system, which has a relatively small footprint and has user-friendly interfaces.

The MagPix uses a ruggedized hardware platform that does not require a company-trained individual to conduct operations. Manufacturer provider performance is provided in Table H-2.

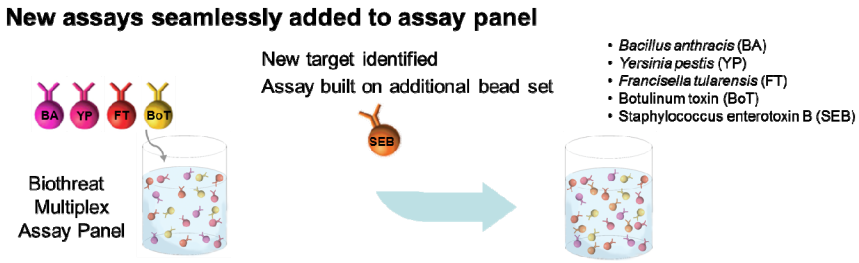


FIGURE H-2 Illustration of the multiplexing capability of existing Luminex antibody-based assays for biothreat targets.

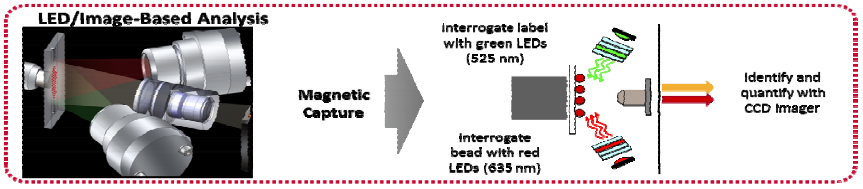


FIGURE H-3 Illustration of the optical imaging system of MagPix.
NOTE: CCD = charged-coupled device; LED = light-emitting diode.

FIGURE H-4 Image of the complete MagPix system with computer interface.

TABLE H-2 MagPix Performance

Multiplexing capability	Up to 50 individual analytes
Reagent compatibility	MagPlex® magnetic microspheres
Dynamic range	3.5 logs
Microplate type	96-well plate
Reading speed	96 wells ≤60 mins
Sample temperature control	35°–60°C (95°–131°F)
Sample volume uptake	20–200 ml
Probe piercing	Yes
Auto adjust-probe height	Yes
Daily startup	≤15 minutes
Sensitivity	Approximately 106 copies of DNA or single-digit picogram levels of protein, 10^2–10^3 copies of bacteria.
Dynamic range	3.5 logs
Strengths	The open architecture allows for significant flexibility for generation of additional tests in any laboratory. Significant field experience and design engineering experience.
Weakness	Only a fraction of the beads incubated with target are analyzed, which dilutes the signal.

PathSensors, Inc.

PathSensors, Inc. (PSI) manufactures reagents and instrumentation for the detection of pathogens in indoor and outdoor environments, with a focus on biodefense and agri-food applications. Detection of pathogens is based on the CANARY® technology originally developed at Massachusetts Institute of Technology's Lincoln Laboratory (*Science*, 2003) and licensed exclusively to PSI for these applications.

The CANARY® technology shown in Figure H-5 differs significantly from the standard immobilized antibody. This is the only technology discussed in this paper that exploits the natural mechanism of antibody response when a specific antigen is detected. The antibodies are expressed in B lymphocytes that are the natural display mechanism within the host. Upon formation of a dimer with the antigen serving as the bridge, the antibodies stimulate a cascade of intracellular events. In this process, the cascade is linked to the generation of photons, which are detected using a photomultiplier tube. The process forces antigen and antibody together by centrifugal force and the response is as specific as the antibody and very rapid. The complete response occurs in less than 5 minutes.

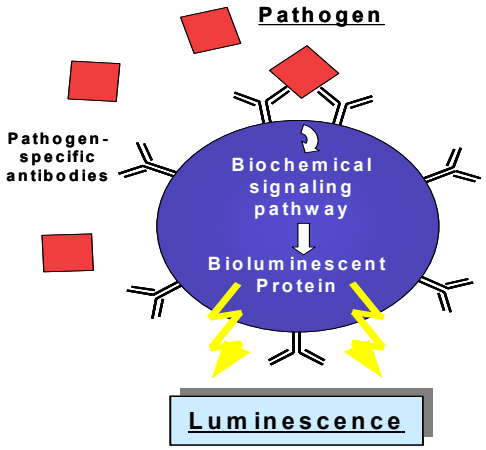


FIGURE H-5 Principle of the CANARY® Biosensor. B-cell lines are engineered to express membrane-bound antibodies that trigger the release of intracellular calcium upon specific interaction with their target. These intracellular signaling processes rapidly culminate in the activation of bioluminescent proteins to generate photons of light within seconds after contact between the biosensor and its cognate pathogen.
SOURCE: Pathfinder CEO.

The principal benefits of the CANARY® technology include the speed of detection, which takes as little as 2 minutes, and the sensitivity that is conferred by the intracellular signal amplification, which allows detection of very low levels of target. In analytical assays, CANARY® demonstrates the ability to detect as few as 10–100 colony-forming units (cfu) of target organisms with a time to result of 3 minutes, including sample processing (see Figure H-6).

This technology has been fielded in one or more Department of Defense buildings for over 5 years with excellent results. The monitoring system operates with a trigger from a particle detector. The system has had thousands of collections and tests without any false positives over the 5-year period of operation. The system is fully capable of adding additional targets by the incorporation of different antibody genes into the progenitor B-cell line. This technology is a definite candidate for BioWatch, either as a trigger or as an integrated detection/identification system.

Strengths: A significant strength of this approach is speed, with results within minutes with good sensitivity.
Weakness: Reagent stability is a concern because the B cells must be viable in order to respond. Anything in the collected sample that harms the integrity of the B cell could cause a problem.

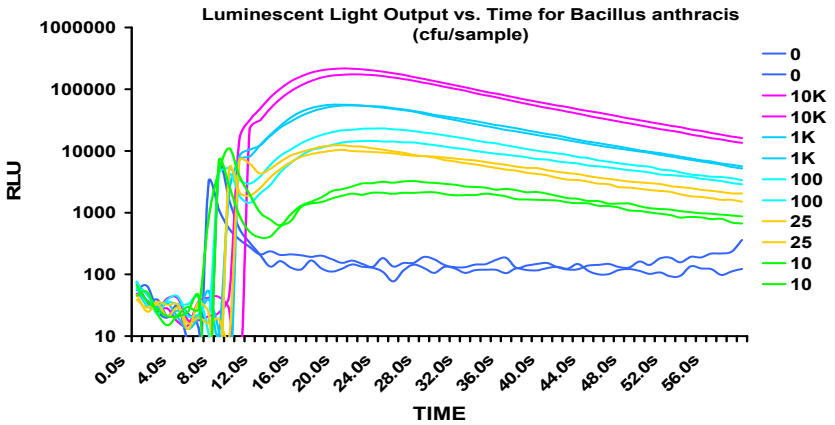


FIGURE H-6 Sensitivity and speed of the CANARY® technology. In analytical assays, CANARY® enables detection of 10 cfu of *B. Anthracis* (Sterne) spores in only 3 minutes.
NOTE: RLU = relative luminescence unit.
SOURCE: Pathfinder CEO.

TABLE H-3 Selected Subset of Available B-Cell Lines with Analytical Sensitivities

Agent	Antigenic Target	Test Panel	Assay Sensitivity
Bacillus anthracis (spores)	Targets anthrax spore coat antigen	Positive against live Sterne strain, gamma-killed and live Ames strain	10 cfu/sample (Sterne) <100 cfu/sample (Ames)
Yersinia pestis	Targets F1 antigen	Tested against inactivated *Yp* CO-92, and live Kim5 strains	50 cfu/sample
Francisella tularensis	Targets cell wall polysac-charide	Positive for inactvated *Ft* Schu4, LVS	<100 cfu/sample
Smallpox	Orthopox specific	Tested against live vaccinia virus, inactivated vaccinia virus (Lister strain)	<100 pfu/sample (UV-Psoralen inactivated virus) 500 pfu/sample (live virus)
Brucella spp.		Tested against *B. suis*, *B. abortus*, *B. melitensis*	50 cfu/sample
Venezuelan equine encephalitis (VEE) virus	Targets VEE virus capsid protein	Positive against killed VEE3880, Menall, P676, TC-83, PTF-39, and Trinidad donkey	500,000 pfu/sample Strain variability in LoD
Ricin	Ricin A chain	Tested against active ricin	<0.4 ng/sample
Botulinum toxin	BoNT/AHc	Tested against active botulinum toxin A	16 pg/sample against active toxin
Bacillus subtilis spores		Tested against live spores	50 cfu/sample

NOTE: BoNT/AHC = heavy chain of botulinum neurotoxin serotype A; cfu = colony-forming unit; LoD = limit of detection; LVS = live vaccine strain; ng = nanogram; pfu = plaque-forming unit; pg = picogram; spp. = species; UV = ultraviolet.

Research International

Research International, Inc. (RI) has been involved in the BioWatch program for a number of years. It supplied a portion of the air sampling system to Lawrence Livermore National Laboratory during development of the autonomous pathogen detection system. Since then it has been a subcontractor to Northrop Grumman on the Gen-2 program and has manufactured the entire aerosol collection system for all the units delivered to date.

The primary operating costs for a BioWatch Program accrue from the biological assays needed to detect and identify pathogens. The assay chemistries required and the labor involved in frequent servicing are expensive. To minimize these costs one could implement a strategy of monitoring bioaerosol concentrations with an optical biofluorescence instrument that has no consumables and minimal maintenance. Only when this instrument detects a rise in aerosol concentration would collection and wet assay identification be initiated.

The TacBioHawk is an integration of the TacBio biological particle detector and the BioHawk aerosol collector and bioidentifier into a single package. It is designed for continuous outdoor monitoring of biothreat agents over a wide range of environmental conditions.

The biological particle detector uses ultraviolet-excited fluorescence to monitor biological particle levels in the air and elastic scattering to monitor other types of particles. This particle detection portion of the TacBioHawk is extremely useful for tracking background levels of airborne materials and providing an alarm if there is an unusual increase in biological aerosol levels. However, it does not identify the type of biological material detected, and for that reason it is correctly characterized as a "trigger."

The BioHawk portion of the system provides identification in the event of a TacBio alarm. When the BioHawk is triggered into operation by the TacBio, an aerosol sample is automatically collected and an eight-agent sandwich format fluoroimmunoassay is performed. The entire process can be completed with no human intervention within a 20- to 30-minute period. Additional samples can be collected and assayed within 17 minutes.

TacBio Biodetector Module

The TacBio aerosol biodetector's mode of operation is based on the well-known fact that non-biological materials primarily absorb and scatter impinging ultraviolet (UV) light, whereas biological materials fluoresce as well as scatter UV light. With the recent development of GaN semiconductor light-emitting diodes, high-brightness UV sources have become available that are energy efficient and more reliable and temperature tolerant than current UV lasers (<400-nm wavelength). In addition, very-high-speed analog electronics are used to count the individual photons emitted by each particle, providing a "Geiger counter" operating mode that eliminates most of the drift and aging problems seen with analog optoelectronics. The TacBio requires little maintenance and no consumables, and it is an ideal first line of defense for monitoring areas that cannot be accessed regularly.

Although aerosol data can be transferred to a remote location for analysis and storage using the TacBioHawk's serial RS-232 data line, it is also stored internally on a removable SD memory card within the unit. This card can store up to 5 years of aerosol alarm data.

BioHawk Module

The BioHawk® is a portable eight-channel bioassay system with automated sample collection capability that is suitable for high-sensitivity monitoring of live agents and protein toxins. Once activated by the TacBio, the BioHawk® collects aerosol particulates from the surrounding air with its wetted wall cyclone air sampler and automatically transfers the concentrated sample from the air sampler to a bioassay module. This module performs automated sandwich-style antibody assays in eight separate detection channels over a period of 15 to 20 minutes, after which results are communicated to users through a touch-screen liquid-crystal display and by wireless or RS-232 link to personnel at a remote location. System operation may also be remotely controlled in real time with virtually every fluid or air handling function being capable of remote manipulation.

The bioidentification portion of the instrument can also analyze fluids from other sample collection systems or from liquid samples loaded into the instrument through a manual sample port. A built-in microcomputer provides unparalleled operating flexibility and sophistication.

Bioassays are performed within a disposable, credit card–sized, plastic assay coupon. Unlike other technologies on the market that are based on a "use once and discard" philosophy, this coupon and the reagents stored within it may be used for 10 assay procedures before being discarded. Because the coupon can execute up to 8 different assays simultaneously, a total of 80 individual assays may be performed before the coupon is discarded. This capability can substantially reduce life-cycle cost.

While possessing a high level of function and great versatility, the unit is still very easy to use. Most global functions such as air sampling and bioidentification are performed using multistep protocols (recipes) developed by RI and stored in the system's computer memory. The unsophisticated user needs only the most fundamental level of training since the internal processes and steps are preset through the built-in computerized recipes. For more sophisticated users, bundled Windows-based software allows the development of customized sample collection and detection protocols.

The system is designed to be operated over a very wide temperature range, from −32°C to 60°C. In low-temperature environments sampled air is preheated to above freezing before entering the wetted wall cyclone sampler. At high ambient temperatures, cooling fans circulate air into the transport case to prevent overheating. Bioassay reagents are maintained in a very stable lyophilized (freeze-dried) form until they are needed and are only rehydrated when a bioassay is to be performed, greatly extending the assay coupon's useful life. Once hydrated, reagents are useable for a period of up to 48 hours.

The current version of the TacBioHawk was developed for the armed forces of a NATO country, which specified using only a limited amount of power for the unit. Because air temperatures below freezing require heating the incoming air for operation of the wetted wall cyclone and for operation of the immunoassays, the air sampler was limited to 40 liters per minute (lpm). However, access to mains power will allow operation with a 325-lpm aerosol collector. If this volume proves insufficient, RI's SASS 4000 aerosol preconcentrator, which operates at 3600 lpm, can be integrated into the system. It would increase overall sensitivity by roughly a factor of 10.

The characteristics of the TacBioHawk are as follows:

- Sensitivity
  - 8×10^4 cfu-min/m^3 for *Bacillus anthracis* spores with 325 lpm aerosol collection.
  - Probability of detection of 95 percent for subspecies dependent on the particular Department of Defense Critical Reagents Program antibodies used.
- Specificity
  - False-positive rate 1 to 2 percent for 17-minute assay with a limit of detection of 5×10^4 cfu/mL for *B. anthracis*.
  - Speciation is dependent on quality of C-reactive protein (CRP) antibodies
- Flexibility
  - Current hardware supports simultaneous assays for one to eight agents in the same 2-mL sample. Addition of a second detection module would increase range to 16 agents. A maximum of 20 agents is possible.
  - Quantitative information is saved in on-board flash memory and may be automatically transmitted to a remote computer if desired.
- Measurement interval
  - Aerosol trigger: 1 minute updates
  - Sample collection and agent identification initiated on alarm from aerosol trigger
  - Sample collection: 5 minutes minimum recommended for first sample after alarm
  - Agent identification: 30 minutes assay time for first sample after first collection
  - Sample collection: user definable after initial collection
  - Agent identification: 17 minutes for each additional sample
- Cost
  - Initial purchase: $125,000 to $150,000 in quantities <10 depending on exact configuration. Significant discounts available for larger volumes.
  - Operational maintenance: <$1,000/year hardware; ~3 hours/quarter staff time for cleaning.
  - Consumables: $250 per assay coupon. Coupon only used if assay is triggered. Once triggered, up to 10 assays may be run.

- Automation
  - Once set up on site, the TacBioHawk is fully automated. Only maintenance and replacement of consumables required.
  - Mean time between failures: >10,000 hours with regular maintenance.
  - Availability: currently 99.9 percent or better depending on environment.
- Operational environments
  - Can operate in dirty internal environments
  - Current design can operate −32°C to 60°C. Operation between 50°C and 60°C is permitted up to a total time of 1,000 hours.
  - Once set up on site, the TacBioHawk is fully automated. Only maintenance and replacement of consumables required.

Strengths: This system has extensive field testing.
Weaknesses: Specificity of antibodies may not provide needed discrimination.

Battelle REBS Biosensor Technology

Battelle has patented an integrated collection and identification spectroscopic technology, the Resource Effective Bio-Identification System (REBS), which provides a portable platform that is a lightweight, low-cost, networked, battery-operable system with near-real-time identification of environmental aerosols, surface contamination, and waterborne biological warfare agents (BWAs) (see Figure H-7). The process uses Raman spectroscopy for BWA identification and has undergone extensive government field testing over several years. By using a spectroscopic method, REBS significantly reduces the amount of consumables and overall life-cycle cost, reduces the amount of time to identify agents, and includes an agile software-updatable identification capability for emerging threats. REBS autonomously processes samples from collection to detection/collection subsystems through to identification, thus removing errors that are often encountered in manual sample processing methods. REBS delivers an archive confirmatory sample via a removable vial compatible with genetic and other confirmatory methods. REBS delivers performance for approximately $0.05 per sample. This drastic reduction in identification cost versus that of traditional systems allows for contin-

uous collection and identification of BWAs while eliminating the burden of reagent usage.

Probability of Correct Identification

REBS demonstrated a probability of correct identification above 97.5 percent for the samples supplied by a government sponsor for an identifier technology readiness evaluation. During limit-of-identification testing at Battelle Memorial Institute, all of the aerosol challenge samples were identified correctly. Based on the number of trials in this test, the 95 percent confidence bounds on the probability of correct identification are 96.5–100 percent. These tests included four BWAs, including gram-negative and gram-positive bacteria, virus, and protein simulants.

Battelle has carried out extensive testing to establish the probability of correct identification, first based on Raman spectroscopy of single particles and, second, on the consensus of several identified particles

FIGURE H-7 Battelle's Resource Effective Bio-Identification System (REBS) provides collector, detector, and identifier functions within a single, portable system.
SOURCE: Provided by Battelle.

within an aerosol collection. The probability of correct identification for a single particle has consistently performed to greater than 90 percent for any agent within an extensive spectral database. However, a system alarm utilizes several particles to form a consensus on the identity of a collected aerosol sample. Based on the consensus result, the system had been configured to identify at least three particles to confirm the aerosol identity. By accumulating information over these three particles, the probability of correct identification for a sample is above 97.5 percent for all agents tested.

Detection and Identification False-Positive Rates

REBS has been extensively tested concerning its detection and identification false-positive rates in realistic environments. The REBS false-positive rate has been measured over several years in both indoor and outdoor locations throughout the United States. In government-sponsored tests, REBS demonstrated 1,629 hours of outdoor operation without a reported false-positive, at Ft. Bliss in El Paso, Texas, during July 2009. To date these tests correspond to 9,774 distinct samples with a 10-minute analysis interval. More recently, three REBS systems were tested in the Boston subway system from October 2012 through April 2013. In this test REBS demonstrated 2,454 hours of operation in the subway system without a reported false-positive. These tests correspond to 5,860 distinct samples with a 15-minute analysis interval.

No samples in any trial have been identified as one of the targets within the system database, and therefore the probability of false-positives currently has an average of 0.0 percent with an upper bound of 0.001 percent (80 percent confidence interval). Naturally occurring biological particulates are routinely detected in these tests, but none has been misidentified as *Bacillus anthracis*, *Francisella tularensis*, Venezuelan equine encephalitis, *Clostridium botulinum* toxin, or any other material within the system database.

Size and Weight

REBS is 1.5 cubic feet and weighs 38 pounds. Its dimensions are 18 × 12 × 12 inches. REBS requires electrical power from at least one source. Potential power sources include military-style batteries, 120- to 240-VAC (volts alternating current) at 50- to 60-Hz (hertz) facility power, or 21- to 32-VDC (vehicle dynamics control) vehicle power.

System Communications

REBS is locally controlled via a simple push-button keypad. Alarms, warnings, battery life, system status, configuration, and meteorological conditions are viewable by a liquid crystal display interface adjacent to the keypad. Remote control and monitoring are achieved using a wired or wireless network communication protocol and remote computer graphical user interface.

Expandable Threat Identification

REBS detects and identifies at the genus, species, and strain levels based on the Raman signatures generated by a laser field interacting with the molecules and structural conformations of biological materials that comprise BWAs. Battelle currently maintains more than 120 spectral signatures in an on-board database, which is easily and cost-effectively expandable to include new threat materials through a simple software patch update via wireless connectivity. As a result, hardware modifications, system recalibrations, perishable assays, and costly reagents are not required to expand the functionality of REBS. Battelle has constructed a library of signatures for various spores, bacteria, viruses, toxins, common battlefield interferants, chemical warfare aerosols, and some explosives. Expanding threat identification signatures takes as little as 24 hours and has been tested in government-sponsored technology readiness evaluations. Once the signature is determined, it can be sent wirelessly to fielded systems, enabling instantaneous enhanced threat warning capabilities.

Analysis Interval

The limit of identification for the REBS system is dependent on the analysis time interval. An increase in the time available for analysis increases the sensitivity of the system. An analysis time of 20 minutes is approximately two times more sensitive than the same system configured for a 10-minute analysis time. The additional analysis time allows for more materials to be collected and more of the sample to be analyzed. The ability to change the REBS duty cycle represents a unique capability; the duty cycle can be adjusted via software configuration changes. This attribute can be desirable for the various situations where the threat likelihood may be higher than normal. In government-sponsored testing, REBS was configured to collect and analyze samples with a 20-minute

duty cycle. In this test REBS demonstrated a 23-minute time to result at a BWA simulant concentration of approximately 200 particles per liter of air.

System Cost and Consumables

REBS is a reagentless BWA identification technology that eliminates the need for expensive and perishable reagents and consumables. REBS is significantly less expensive to operate than any other equivalent technology, based on logistical burden expense reduction alone. Moreover, the REBS ability to add new threat agents via software offers a compelling value over similar technologies that are assay- or wet chemistry–based.

Strengths: Cost per analysis is low, rapid cycle time, nondestructive analysis of sample.
Weakness: Customers are not familiar with technology, therefore a significant barrier to entry.

MesoScale

The MesoScale technology is a mature system with many systems in operation. The electrochemiluminescence system offers significant sensitivity and was once a system of choice for the U.S. Army. The system provider was purchased, and the older systems are no longer fully supported by the new owner. Antibody-based assays immobilize the capture antibody in a black plastic well, and then standard sandwich assays are performed with a proprietary reporter light-generating reaction.

Electrochemiluminescence detection uses labels that emit light when electrochemically stimulated (see Figure H-8). Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light).

Labels are stable and nonradioactive and offer a choice of convenient coupling chemistries. They emit light at ~620 nm (nanometer), eliminating problems with color quenching. Few compounds interfere with electrochemiluminescent labels, so one can use large, diverse libraries with confidence. Multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity.

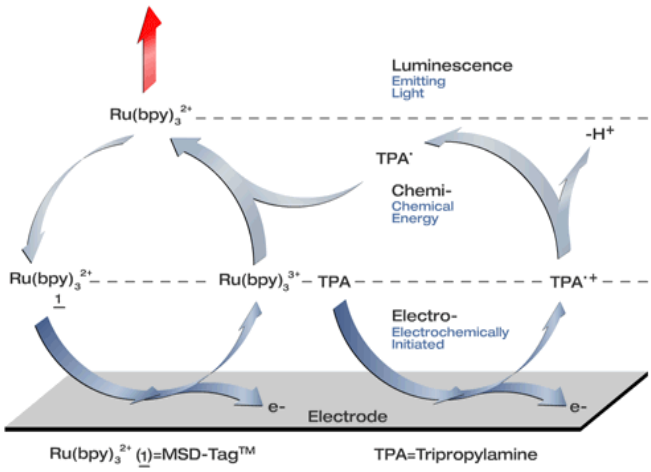


FIGURE H-8 Representation of the electrochemical light-generating reporter system for MesoScale technology.
NOTE: MSD = Meso Scale Discovery; Ru(bpy) = ruthenium tris difulleride; TPA = tripropylamine
SOURCE: Company website.

FIGURE H-9 Multispot plates, which offer arrays within the well for increased throughput and assay multiplexing, are available in 24-, 96-, and 384-well formats, with up to 100 spots per well.
SOURCE: Company website.

The system offers multiplexed detection technology by spatially separated spots on the bottom of a 24-, 96-, or 384-well format microtiter plate (see Figure H-9).

Sensitivities of the MesoScale systems are generally superior to standard ELISA assays. The sensitivities achieved by currently available systems are adequate for the BioWatch requirements.

Strengths: Sensitive reproducible tests. Proven technology.
Weakness: Not currently configured for BioWatch operations.

TECHNOLOGY FOR FUTURE APPLICATIONS

Quanterix Corporation Simoa Technology

Quanterix has developed, and is commercializing, a novel antibody-based technology that makes some fundamental changes when compared with existing approaches. The figure below provides a cartoon diagram of the various steps from sample collection to readout of results.

Antibodies are covalently attached to microspheres and then incubated with sample in such a way that the ratio of bead to target is slightly less than unity. The goal is to have only one target per bead. All reactions are in solution phase, which speeds the binding reaction compared to antibodies fixed on a planar surface. The beads occupied with target are then incubated with a secondary reporter antibody, which is covalently linked to an enzyme.

The conjugated enzyme bead complex is rinsed in a solution containing an optically inert substrate. The bead–substrate mixture is floated across a plane of microwells, each of which can hold only one bead. The wells are then sealed and the enzyme converts substrate into fluorescent product. Each fluorescent well represents a single target molecule. The process is diagramed in Figure H-10. The result is a quantified number of targets with sensitivities up to over 1,000-fold demonstrated with existing technology.

The current instrument, pictured in Figure H-11, is a floor standing unit.

While the biodefense industry may not be a current market for Quanterix, the technical approach offers some significant improvements over existing technologies. This type of approach should be considered for post-2020 fielding.

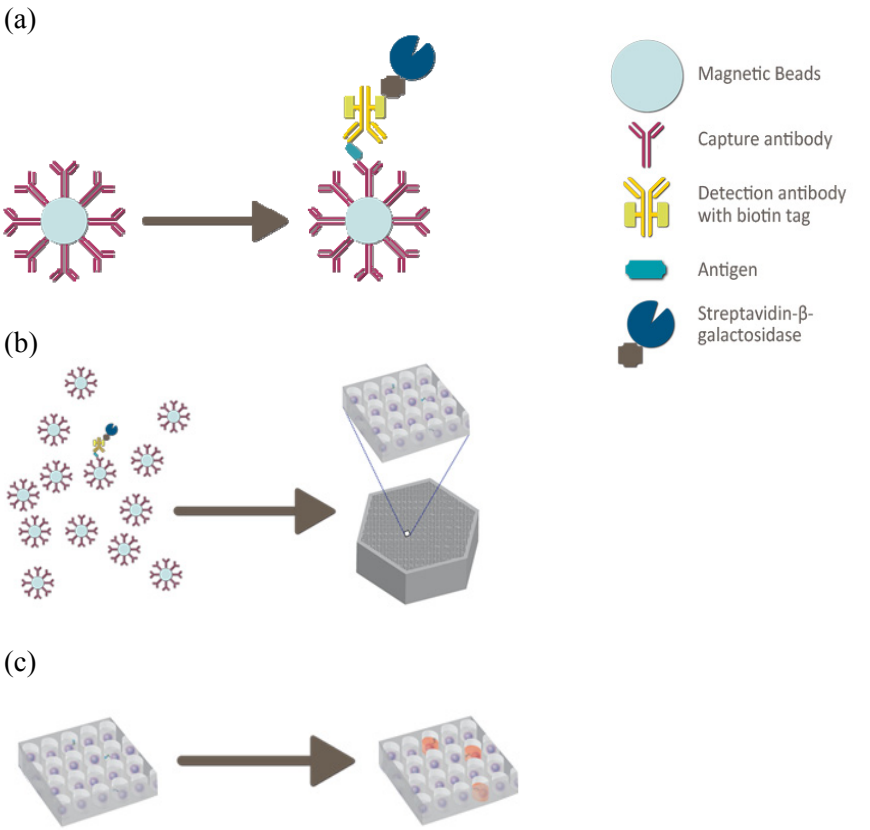


FIGURE H-10 The Quanterix antibody-based technology. (a) Single protein molecules are captured and labeled on beads using standard ELISA reagents. (b) Tens of thousands of beads—with or without immunoconjugate—are mixed with enzyme substrate and loaded into individual femtoliter-sized wells. The microwells are sealed with oil. (c) Fluorophore concentration in the small sample volume of wells containing the target analyte rapidly reach detectable limits using conventional fluorescence imaging and can be digitally counted. The percentage of beads containing labeled immunocomplexes can be computed at low concentration because they follow a Poisson distribution; at higher concentrations the intensity of the aggregate signal provides an analog measurement.
SOURCE: Company website.

FIGURE H-11 Quanterix Simoa HD-1 analyzer.

SUMMARY

All of the systems presented have the capability to meet the majority of the autonomous detection system requirements. The most significant challenge will be the ability to provide sufficient specificity for organism discrimination. A clear example of this is that a Food and Drug Administration–approved antibody-based test for *Bacillus anthracis*, sold by Tetracore, Inc., uses the harmless vaccine strain for a positive control. This challenge may be overcome by the speed in which these technologies provide information to the customer community.

I

State of the Art for Autonomous Detection Systems Using Genomic Sequencing

John Chris Detter, Ph.D., and I. Gary Resnick, Ph.D.

A white paper prepared for the June 25–26, 2013, workshop on Strategies for Cost-Effective and Flexible Biodetection Systems That Ensure Timely and Accurate Information for Public Health Officials, hosted by the Institute of Medicine's Board on Health Sciences Policy and the National Research Council's Board on Life Sciences. The authors are responsible for the content of this article, which does not necessarily represent the views of the Institute of Medicine or the National Research Council.

BACKGROUND

The BioWatch Program at the Department of Homeland Security (DHS) was developed and is currently operating to provide warnings of aerosol attacks with biological threat agents. A select number of urban centers have had BioWatch deployed for a number of years on a round-the-clock basis. These deployments have provided a great amount of operational experience that indicates great need for technology improvement. While the current BioWatch capability provides an important risk mitigation capability against biological warfare and terrorist (BW/BT) threats; many operationally significant challenges remain to be addressed. A brief description of the desired capability, with extant challenges, is presented below to provide a framework for discussion of technical approaches employing sequencing to address current technology limitations.

Affordable Continuous Coverage of At-Risk Populations

The population of the United States is dispersed across a large landmass. People converge to great population densities at numerous venues for varying lengths of time (e.g., day/night workday cycles between cities and suburbs and special-event gatherings). Therefore, BioWatch must cover a large geographic area and varied indoor structures (e.g., special event centers, office buildings, transit centers, and underground rail systems). To achieve this in a sustainable manner the capital and annual operating costs of the system must be commensurate with the assessed relative risk from BW/BT and the myriad of other needs faced by local, state, and federal officials.

The current BioWatch system uses field aerosol concentration and sample collection, followed by the transport of samples back to a central laboratory and laboratory analysis of the samples. BioWatch data outputs are provided to a decision-making body for an integrated analysis prior to taking response actions. Major decreases in the resources required for BioWatch as well as improvements in the overall efficiency and effectiveness can be achieved through technical advances that provide for field in situ detection and identification (an autonomous deployed detector) to eliminate sample transport and laboratory analysis costs; amplification-free nucleic acid detection to decrease reagent costs; reagent-free detection to decrease reagent costs, eliminate the need for environmental engineering controls, and minimize the need for electrical power for the deployed autonomous detector; inexpensive analysis of agent recognition events within the autonomous detector to decrease sensor unit production cost and maintenance; and system modularity to minimize technology refresh costs.

Accuracy and Precision Supporting High-Regret Responses

Surveillance derives its value by informing response management systems that have the potential for eliminating or mitigating the impacts of risks. Response options vary in efficacy, cost, and associated negative consequences. There are also negative impacts associated with false-positive system outputs. Therefore, the accuracy and precision of the BioWatch system have a profound impact on overall system performance, value, and sustainability.

Operational experience with the current BioWatch system indicates a strong need for improved accuracy while maintaining robust precision.

The great diversity of the microbial world coupled with the fact that only a fraction of this diversity has been identified and characterized has resulted in a significant number of "environmental positives" that have adversely impacted BioWatch performance. The fact that no aerosol attacks have been detected and that no impacts of such attacks have been observed provides little evidence for understanding the false-negative potential. This is greatly compounded by the inherent uncertainty of the biothreat.

Responsiveness to Full Scope of Biological Warfare and Terrorist Threats

The uncertainty associated with the biothreat (e.g., which agents will be encountered at which locations, when it will occur, how much will be delivered, and how it will be dispersed) provides great operational constraints on BioWatch. A large number of detector units are needed to cover populations at risk, and the system should be responsive to all potential biothreat agents (including emerging, re-emerging, and engineered pathogens) that may be presented as aerosol threats. In addition, an indication of unique agent phenotypic characteristics is desirable in order to guide response decisions (e.g., whether the specific strain encountered is responsive to a particular antibiotic).

The current BioWatch system is focused on a specific set of pathogens and provides some level of identification. To be fully responsive to the potential bioaerosol threat, the scope of agents addressed must be greatly increased and the cost of coverage must be drastically decreased.

CURRENT STATE OF SEQUENCING

Next-generation sequencing (NGS) platforms have remarkable performance specifications. Most of them produce very high quality data in an automated or semiautomated fashion. Some are small, benchtop models, able to produce large amounts of data in a relatively short time and for a relatively low cost. The rapid advancement in NGS technologies will soon enable pathogen detection devices to rely on sequencing to provide a wealth of information about the environment in a cost-effective and timely manner.

NGS technologies can be used to sequence almost any sample containing biological material, such as clinical (human, animal), environ-

mental (water, soil, surfaces, plants, etc.), or pure cultures of organisms of interest. Sequencing can include DNA, RNA (in the form of cDNA), or both, depending on the type of information needed. For example, DNA sequencing can reveal which organisms are present in a sample and some of their phenotypic characteristics (e.g., antibiotic resistance). However, RNA sequencing (RNAseq) must be used for RNA viruses. RNAseq is also used to study the transcriptional profile of organisms at specific time points, allowing for a better understanding of their metabolic activity and for identification of genes that play key roles in disease, genetic disorders, inflammatory response, cancer, and so on.

NGS technologies require that DNA molecules are converted to NGS libraries. RNA is always converted to DNA first, as currently there are no direct RNA sequencing technologies (although the potential exists with the Pacific Biosciences (PacBio) real-time sequencer (RS), which is not evaluated here because of its very large footprint). Standard library preparation processes include DNA fragmentation and the addition of appropriate adapter molecules to the ends of DNA fragments. Adapters are unique DNA sequences (usually 30 to 60 base pairs long), which allow sequencing to occur, and they can also incorporate barcodes (or indices) that enable analysis of many samples in parallel. Depending on the application, library preparation methods take between 2 hours and 3 days. Once the libraries are prepared, each DNA fragment present in the library is clonally amplified before sequencing. This process and its degree of automation depend on the NGS platform. For example, the Illumina platforms use clustering (MiSeq is fully automated), whereas 454 and IonTorrent platforms use emulsion polymerase chain reaction (PCR) techniques (which will soon be mostly automated on IonTorrent).

NGS produces vast amounts of data, which are output as reads. A read is a string of DNA nucleotides corresponding to the sequence of the original DNA or RNA molecule in the sample. Each NGS platform outputs reads with three important characteristics for interpretation: read length, the number of reads, and their quality (fidelity). Read lengths vary from less than 50 base pairs (bps) to >3,000 bps, depending on the platform and sequencing kits. Read numbers vary from as few as 1.5 million to 4 billion per run. Reads can be evaluated independently, or they can be combined into much longer strings of DNA or RNA sequences using a variety of computational tools.

Sequencing of any sample generally requires four steps: nucleic acid extraction (sample preparation), library preparation, sequencing, and data analysis. Figure I-1 outlines a standard NGS workflow. Sequencing can

be performed on any one of the current NGS platforms, with each having different requirements and often their own unique set of data output and management. Table I-1 briefly lists the facilities, personnel, equipment, software, and reagents needed for each platform.

For many applications, sequencing offers great advantages over the traditional methods. For example, in the field of pathogen detection, NGS can identify not only known organisms but also indications of novel, emerging, and engineered ones. Comparative analysis to known pathogens, the presence of virulence genes, and recombinant engineering markers and phylogenetic placement would provide indication of novel threat agents. This is highly relevant, especially for rapidly evolving and highly diverse organisms, such as RNA viruses and *Burkholderia* spp. In addition, NGS does not require prior knowledge of pathogens present in a sample as do the traditional detection methods. Therefore, NGS shows promise as the ultimate pathogen detection tool. Other application areas in which NGS will play a significant role include pathogen characterization (strain typing, antibiotic resistance, etc.), bioforensics, biometrics, and biosurveillance. For NGS to succeed in all these applications, basic studies and databases that correlate genotype (genetic sequence of an organism) to phenotype (the behavior of an organism, such as its pathogenicity, transmissibility, resistance, etc.) are required. Without bioinformatic analysis, the data cannot be used to make these determinations and inferences.

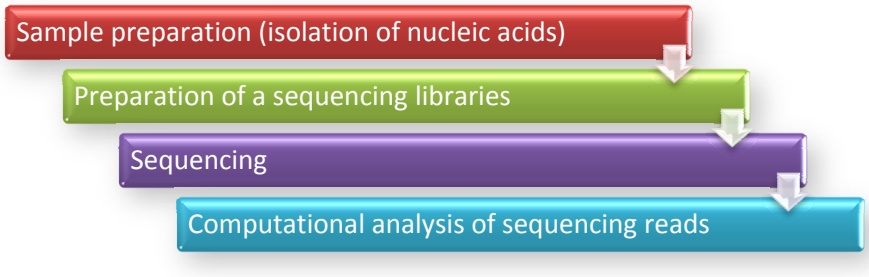


FIGURE I-1 Overview of the next-generation sequencing process.

TABLE I-1 Characteristic Differences Between Sequencing Platforms

Sequencer	Facilities and Equipment	Personnel[a]	Software and Hardware	Reagents
MiSeq–Illumina	68.6×56.5×52.3 cm (W×D×H). Total weight: 57.2 kg. Suggested lab bench space about 1.5× that size available prior to installation. The laboratory must be maintained at 22±3ºC for proper functioning. Can run on standard electrical systems.	One to two individuals are needed for sample receipt through sequence data generation. One or two individuals are suggested for data interpretation.	Instrument includes all software required through data generation. Analytical tools are available from multiple sources and can utilize platforms from a PC laptop through a large server system.	Library preparation and sequencing run reagents are available through Illumina and can be ordered via phone or the Internet. Alternate library preparation kits are offered through multiple scientific supply vendors.
Roche454	Full size Roche454 GS FLX+ Upper assembly • 74.3×69.8 ×36.1 cm (W×D×H) • Includes an 82.5-cm monitor Lower assembly • 75.2×90.8 ×92.7 cm (W×D×H) Total weight: 242 kg. Can run on standard electrical systems. GS Junior 40×60×40 cm (W×L×H) Total weight: 25 kg. Can run on standard electrical systems, also	One to two individuals are needed for sample receipt through sequence data generation. One or two individuals are suggested for data interpretation.	Includes sufficient software for data generation and a multitude of tools exist for data analysis and assembly. The full-size sequencer is well suited to computational systems ranging from large desktop PCs to servers. GS Junior may be analyzed with less computational power, however (as with analyzing any NGS data on such a small	All standard library preparation and sequence run reagents can be purchased directly from Roche and integrate well with the platform. Orders can be easily placed via phone or Internet.

	able to operate between 85 and 264 VAC.		system), the time required may be substantial.	
IonTorrent	61×51×53 cm (W×D×H). Total weight: 30 kg. Operates best between 20 and 25ºC with a humidity of 40–60%. Optimal elevation is below 2,000 m (LANL has successfully operated instrument at >2,200 m). The instrument runs on 100- to 240-V power (standard electrical system) and requires 35 to 45 PSI argon gas for operation.	One to two individuals are needed for sample receipt through sequence data generation. One or two individuals are suggested for data interpretation.	Includes a server to support primary analysis through output file generation and variant calling. Additional processes can be handled through "apps" available through the plug-in store. PGM supports cloud-based processing, much of the analysis can be done from any Internet-linked portal, not requiring on-site computational hardware.	Library preparation and sequencing run kits can be purchased directly through LifeTech, the vendor (phone or Internet sales both supported). Library preparation reagents other than those sold by LifeTech are also commonly used and can be purchased from a variety of scientific supply companies

[a]Separate personnel required for sequence generation and sequence analysis due to the highly specific training required for each skill.

NOTE: When comparing the current NGS technologies consisting of Illumina, IonTorrent, PacBio, and 454, each technology has its own strengths and weaknesses, including the cost of sequencing. If sample and library preparation processes are excluded (they are relatively similar), the cost of sequencing a mega base pair (Mbp) of DNA on each sequencing platform is as follows: Illumina MiSeq, $0.13/Mbp; IonTorrent PGM, $0.57/Mbp; PacBio, $1.40/Mbp; and 454, $6.7/Mbp.

NOTE: FLX = flexibility; GS = genome sequencer; LANL = Los Alamos National Laboratory; m = meters; NGS = next-generation sequencing; PGM = personal genome machine; PSI = pounds per square inch; VAC = volts alternating currents.

SOURCE: Adapted from 2013 Los Alamos National Laboratory sequencing report to Department of Defense.

The promise of NGS cannot be realized without significant investments in the analysis of the produced data, typically referred to as bioin-

formatics. Analysis of NGS data is highly specialized, depending on the types and detail desired from a particular analysis. For the identification and characterization of a pathogen, there are several levels of analysis possible, each with different requirements and able to reach more or less detailed conclusions. For the BioWatch application it may be of value to have two levels of analysis available. The first would be a simple percent match to selected microorganisms that is automatically performed. The second would be an in-depth genomic analysis that would be performed as required.

In conclusion, NGS shows promise for improving current microbiology, molecular biology, and analytical biochemistry methods and for providing new data streams that will help us understand the current state of pathogens and anticipate future changes in the microbial world.

OVERCOMING THE SHORTCOMINGS OF THE CURRENT BIOWATCH SYSTEM

Next-generation sequencing will soon become the ultimate tool for pathogen detection and characterization in clinical and environmental samples. Until recently, NGS was a slow and costly process. However, it is becoming cost-competitive and sufficiently rapid for many applications. Even though NGS is unlikely to replace the current rapid and portable pathogen detection platforms in the next couple of years, in many cases it will provide actionable information faster than the rapid systems. This is mainly due to the comprehensive information provided by an organism's entire sequence versus a few selected segments of the genome. It is the only technology that can perform all of the following tasks in parallel from almost any sample: (1) detect all known pathogens, including viruses, bacteria, and protozoa; (2) identify emerging pathogens, whether they have evolved naturally or been engineered; and (3) characterize the pathogens (e.g., determine antibiotic resistance or pathogenicity).

Over the next 2 to 3 years NGS applications will likely help generate a world map displaying the real-time status of all infectious diseases. The data will be provided by a global network of interconnected facilities that use NGS platforms. Sequencing data, combined with the computational models of disease progression and easy visualization, will enable the accurate prediction and monitoring of disease spread and will reduce the effects on human lives and local economies.

With the existing or forthcoming hardware and software upgrades, NGS technology will provide actionable information in 8 to 48 hours (including sample preparation, analysis, and interpretation), depending on the platform, the number of samples, and types of information needed. The simplest process includes detection of known pathogens and determination of some of their features, such as antibiotic resistance. More complex processes will involve identification of novel pathogens in mixed samples (clinical or environmental samples such as BioWatch aerosol samples), prediction of their pathogenicity and susceptibility to antibiotics, vaccine efficacy, and matching their identities to pathogens that previously caused serious outbreaks.

The major types of sequencing data can generally be obtained with three different pipelines, each providing different amounts and types of information, depending on the user's requirements (see Table H-2).

It is the only technology that can perform all of the following tasks in parallel from almost any sample:

- Sequencing provides complete genomic picture of all microorganisms, not dependent on a priori selection of a small number of agents.
- Phenotype can be predicted from the sequence, providing response guidance, such as which antibiotics to use and whether existing diagnostic tests will work.
- Organisms altered by genetic engineering can be identified, providing coverage for the engineered threat.
- Previously unidentified pathogens can be presumptively identified by comparative analysis, as was done with SARS and novel coronavirus. This and the previous point should decrease false-negative issues.
- The more in-depth information provided by a draft sequence should give greater accuracy, decreasing false-positive issues and facilitating response decision.
- Can sequencing be done in a reagent-free system? If so, it would eliminate the need for reagents and environmental control, which will radically decrease cost.
- Sequencing can achieve single organism’s recognition, providing extreme sensitivity without compromising accuracy.

Below, we will attempt to address many of the current and future needs of the BioWatch Program as we understand them in key areas im-

TABLE I-2 Overview of the Three Next-Generation Sequencing Pipelines for Pathogen Detection and Characterization

Pipeline	Description	Actionable Information
1. Amplicon Sequencing	Rapid sequencing of very small portions of pathogen genomes	Identify and characterize known pathogens, and some emerging ones. Able to test hundreds of samples in parallel.
2. Pathogen identification and characterization in mixed samples	Full sequencing of environmental and clinical samples	Identify and characterize known and emerging pathogens, including bacteria, viruses, and protozoa.
3. Pure-culture (isolate) whole-genome sequencing	Whole-genome sequencing of one pathogen isolated from a sample and grown in the lab	Can identify sequences associated with specific outbreaks. Allows rapid detection of the same pathogen in future outbreaks.

SOURCE: Adapted from 2013 Los Alamos National Laboratory sequencing report to Department of Defense.

portant to its mission. The information content generated from today's sequencing technologies already provides the base of what BioWatch needs. The challenge is to engineer a fieldable pathogen-detection platform based on nucleic acid sequencing.

Sensitivity

There is high confidence that a robust data stream will be generated from any sample type. Successful sequencing has been achieved at the level of a single bacterium. In addition, metagenomics and ancient DNA sequencing have also been successfully used for identification of small amounts of microorganisms. One drawback of sequencing all of the DNA (and/or RNA) in a sample is that a small amount of pathogen DNA can be overwhelmed by the background, so clever enrichment strategies may significantly increase the sensitivity. For example, some of the current NGS platforms isolate and amplify individual nucleic acid segments in oil vesicles leading to clonal nucleic acid samples for analysis. Future generations of sequencing technologies are expected to achieve even better sensitivities.

Specificity

This is one of the greatest strengths of sequencing, which should eliminate most false-positive and false-negative results. Thousands of sequencing centers around the world are sequencing various environments for many different reasons. These activities are generating a valuable knowledge base that will keep growing at no expense to BioWatch, which will improve the specificity over time even further. Comparative analysis of novel strains of microorganisms will be robust and automated due to the availability of comprehensive microbial databases and analytical algorithms.

Size and Functionality

This is currently an issue due to how the devices were built and to the audiences they have been built for. Currently the health care industry is driving the technology to a smaller, less complicated device for the benchtop in a diagnostic laboratory. With proper motivation and customary engineering designs, an even smaller platform can be built (i.e., look at the progress Apple is making on a regular basis to miniaturize for their market). Commercial drivers will push the industry without much investment from the BioWatch community. However, properly placed motivation will drive the technology to where BioWatch needs it to be in a timely fashion. The unique needs of an autonomous detector (e.g., extended mean-time-to-failure, multitier analyses outputs, remote data transmission, viability assessment) could be identified and shared with interested sequencing platform developers to establish collaborative research and development initiatives.

Flexibility

Multiplexing with standard protocols is a strength of the current sequencing platforms and will likely remain so for future devices. Computational adjustments allow for the ultimate flexibility in NGS. Identification from a mixed or complex sample (i.e., from a filter) is also a strength of sequencing, where one allows the sequencer to generate baseline data on everything that is in the sample and relies on automated bioinformatics tools to sort out the details. This is commonplace today, and capabilities will be even stronger as more data are generated and as computational advancements occur. Community or metagenomic analysis

will decrease the sample processing requirements as well as provide an additional characterization modality. Suspect segments of nucleic acid will be assessed in the context of all nucleic acid in the aerosol sample, providing the opportunity to detect culture media constituents not normally found in ambient aerosols.

More Than Red/Green Decision Making

The information content of sequencing and analysis is so high that not only does one get identification down to the strain, but the confidence in this call is usually very high, even with today's technology. The information content also includes identification of novel and engineered organisms by comparative and phylogenetic analysis. Traditional recombinant vectors can also be targeted to detect engineered pathogens. Results can be generated automatically from raw data using analytical tools and then presented at multiple levels of complexity for BioWatch technical, scientific personnel, and public health decision makers.

Measuring Timelines

Speed and process flow are enhanced for sequencing because one can gather small amounts of target for sequencing and because sensitivity is high by nature. Databases can also be pared down to do rapid on-board detector analyses. Capabilities are currently in hand to develop two levels or modes of analysis (Defense Threat Reduction Agency [DTRA] is developing one currently called EDGE). One can imagine mode 1 in which rapid analysis and identification is done on board the detector via a laptop-sized platform which looks at a pared-down database of pathogens and near neighbors. The second stage would involve the data being remotely ported to a larger comparative analysis server farm capable of doing a much more extensive analysis and confirmatory target identification. This greater level of analysis would support the BioWatch Actionable Result assessment process involving multiple participants.

Measuring Interval

Sequence throughput can allow for large batches of samples to be processed regularly. Technology will allow for an engineering decision to be made as needed in device or analysis design. One will likely be able to choose between a few samples at a faster throughput and at a

higher cost (rapid mode) or a multiplexed process that is slower and has higher content (detailed mode). In doing this today, one may use amplicon sequencing of fewer primer pairs in rapid mode versus many primer sets in detailed mode. In tomorrow's version one could do the same with sequencing a few samples at a time in rapid mode versus the detailed mode where the device would run one large, highly multiplexed run per day.

Cost

Initial investment on sequencing is relatively high compared with some of the other detector platforms. The payoff is in the throughput, single-stage analysis and associated need of less manpower. Future advancements will become even cheaper due to reagentless sequencing.

Automation

Sample preparation and sequencing are highly automatable. However, technical challenges exist due to the current reagent-based platforms. Next-generation, reagentless platforms will allow for a much more automatable system. The health care industry is highly focused on this issue, and it will help drive the mean time between failures down substantially.

Operation Environment

Current technology is aimed at the clinical laboratory and a standard research laboratory setting. As markets drive the development of smaller and more fieldable devices, the upcoming next-generation systems will be much more adaptable to field autonomous outdoor detection. Again, reagentless-type devices will naturally progress in this direction.

Overall, the health care and related technology industry has a desire to overcome many of the hurdles the BioWatch community is also focused on. These advances will happen naturally and at little to no expense to the BioWatch community. However, properly placed motivational tools and collaborations from the BioWatch community to NGS industry leaders will promote a quicker advancement aimed more precisely at BioWatch's mission. Below we generally discuss the three phases we see the evolution being focused.

READINESS LEVELS

The Institute of Medicine provides three readiness levels for a BioWatch autonomous biodetector against which to assess sequencing technology. These levels are as described below. It was assumed that the engineering, testing, and fielding of an autonomous detector would require at least 2 years after the required components (TRL 4) were available.

- Tier 1: fully automated biodetection system, capable of 24/7/365 unattended outdoor and indoor operation, that will be at a technology readiness level of TRL 6-plus by 2016.
- Tier 2: similar requirements, but will not reach a TRL 6-plus level until sometime between 2016 and 2020.
- Tier 3: technologies that have the potential of meeting or exceeding the BioWatch requirements, but a fully automated, TRL 6-plus system would take us beyond the 2020 time frame. For these technologies, describe the current critical paths ("long poles") in meeting the BioWatch requirements and how they might be addressed.

Tier 1

One way to achieve a Tier 1 system would be to expedite the engineering of an available NGS technology for high-throughput amplicon sequencing to create an autonomous field-deployable biodetector for the BioWatch system.

This capability would allow inexpensive, rapid, and very detailed analysis of many samples in parallel. The parallel-processing capability could be used for analyzing multiple aerosol samples, providing narrower time cuts for sampling or numerous distinct amplicons. Currently available and validated primer sets can be easily utilized in this approach, but many others can be added. Amplicon sequencing can detect many known pathogens and their phenotypic features (antibiotic resistance markers and virulence factors). It can also detect and characterize some emerging pathogens, but this capability is limited.

Summary of features:

- Detects all known pathogens, antibiotic resistance markers, and virulence factors.

- Uses multiplexed end-point PCR to generate amplicons that are directly sequenced.
- Can sequence hundreds or thousands of amplicons.
- Has limited ability to identify emerging threats, especially RNA viruses.

A fully automated platform should be smaller than 24 ft^3, should use a regular 110-V outlet, and should automatically analyze samples every 8 hours following sample collection.

Tier 2

A Tier 2 system could be achieved by using improved NGS technology to provide for metagenomic sequencing and analysis. This capability would sequence all nucleic acids present in a sample. It would detect and characterize not only the known pathogens but also most emerging ones. Because small amounts of a pathogen in a large amount of background would severely compromise the platform sensitivity, a strategy for removal of environmental (non-informative) nucleic acids may be desirable or required. Since all NGS platforms require library preparation, sample-to-result time would still be 6 to 10 hours following sample collection.

Summary of features:

- Detects all known pathogens, antibiotic resistance markers, and virulence factors.
- Can detect many emerging pathogens, including engineered ones.
- Even with improved sequencing speeds, the requirement to prepare libraries slows the overall process down.

A fully automated platform should be smaller than 24 ft^3, should use a regular 110-V outlet, and should automatically analyze samples every 8 hours.

Tier 3

A Tier 3 system could be achieved through using a future sequencing technology (i.e., Oxford Nanopore or Gen2 PacBio) to rapidly perform metagenomic sequencing of environmental samples. This approach

would have similar capabilities to the Tier 2 platform, but the sample-to-result times would be much shorter—approximately 1 hour. No library preparation would be required, and the sequencing itself would be much more rapid. The soon-to-be-released Oxford Nanopore technology is expected to offer such a technology advance.

Summary of features:

- Detects all known pathogens, antibiotic resistance markers, and virulence factors.
- Can detect many emerging pathogens, including engineered ones.

A fully automated platform should be smaller than 3 ft^3, should use a regular 110-V outlet, and should automatically analyze a sample in 1 hour (in addition to air sampling, which can vary depending on the application). Reagent requirements should be minimal.

SUMMARY

Sequencing technology driven by strong and diverse markets has and will continue to make rapid advances. In addition, application of existing technologies is enabling rapid growth of genomic databases and filling in the microbial tree of life. The decrease in cost and increase in functionality of sequencing technology, coupled with publically available molecular databases, should drive a growing interest in genomics for decades. Major points to consider in assessing the utility of sequencing to the BioWatch mission are as follows:

- Great market pressure will facilitate advances in sequencing that can be adopted by BioWatch. In particular, research and development for capabilities to satisfy point-of-care and field analysis capabilities will drive development of core sensor components of direct value to BioWatch.
- Tier 1 and Tier 2 timelines would benefit from comprehensive information provided by NGS, but the engineered autonomous detector would continue to require environmental controls and electrical power. Reagent costs could be decreased and have an analysis time of less than 10 hours following sample collection. Complexity of system fluidics would require routine maintenance.

- Tier 3 capabilities hold promise for eliminating reagents and minimizing the need for environmental controls. This would provide the benefits of information derived from sequencing while decreasing the costs associated with reagent systems. Again, a sequencing and analysis time of less than 10 hours following sample collection should be possible.
- An acquisition strategy for DHS should be considered that fosters research and development of specific technology components within the private sector, toward common desired capabilities.

In summary, the inherent information content from sequencing and analysis should meet all the needs of BioWatch, addressing false-positive and false-negative issues. However, for at least the Tier 1 and Tier 2 timelines, significant engineering challenges will have to be overcome to adapt the existing reagent-based systems into an autonomous biodetector with desired attributes. Fortunately, extensive investments are being made by many public- and private-sector entities to develop technology that should provide the core sensor technology meeting the Tier 3 timeline and system requirements.

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State of the Art for Autonomous Detection Systems Using Mass Spectrometry

Peter Snyder, Ph.D., and Rabih E. Jabbour, Ph.D.

A white paper prepared for the June 25–26, 2013, workshop on Strategies for Cost-Effective and Flexible Biodetection Systems That Ensure Timely and Accurate Information for Public Health Officials, hosted by the Institute of Medicine's Board on Health Sciences Policy and the National Research Council's Board on Life Sciences. The authors are responsible for the content of this article, which does not necessarily represent the views of the Institute of Medicine or the National Research Council.

Mass spectrometry (MS) is being considered as a candidate for the Tier 1, Tier 2, and Tier 3 autonomous detection systems. Candidate status depends on how the figures of merit compare to the given desirable characteristics. Samples to be analyzed include a pathogenic and simulant spore former and a pathogen and simulant vegetative cell bacterium.

BACKGROUND

Mass Spectrometry

The mass spectrometer is an instrument designed to separate gas-phase ions according to their mass-to-charge ratio, *m/z*. The heart of the mass spectrometer is the analyzer. This element separates the gas-phase ions. The analyzer usually uses time-of-flight, quadrupole, ion trap, or a

combination of all three to move the ions from the region where they are created to a detector, where they produce a signal that is amplified. The *m/z*, and not only the mass, is of importance. A mass spectrometer can measure the mass of a molecule only after it converts the neutral molecule to a gas-phase ion. To do so, it imparts an electrical charge to molecules and converts the resultant flux of electrically charged ions into a proportional electrical current that a data system converts to digital information, displaying it as a mass spectrum.

Ions can be created in a number of ways:

- Laser ablation of a compound dissolved in a matrix on a planar surface by matrix-assisted, laser-desorption ionization (MALDI).
- Interaction with an energized particle or electron such as in electron ionization.
- Electrospray ionization (ESI) where the eluent from a liquid chromatography (LC) system receives a high voltage, resulting in an aerosol of ions.

ESI is useful in producing ions from neutral macromolecules, because it overcomes the propensity of these molecules to fragment when ionized. ESI is advantageous over other atmospheric pressure ionization processes (e.g., MALDI) because it may produce multiply charged ions, effectively extending the mass range of the analyzer to accommodate the kilodalton to megadalton orders of magnitude observed in proteins and their associated polypeptide fragments.

ESI is a so-called soft ionization technique since there is very little fragmentation of the parent compound. This can be advantageous in the sense that the molecular ion (or, more accurately, a pseudo-molecular ion) is almost always observed; however, very little structural information can be gained from the simple mass spectrum obtained. This disadvantage can be overcome by coupling ESI with tandem mass spectrometry (MS-MS).

The development of ESI for the analysis of biological macromolecules was rewarded with the Nobel Prize in chemistry to John Bennett Fenn in 2002.

The analyzer is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield. MS-MS is the combination of two or more MS experiments. The aim is either to get structural information by fragmenting the ions isolated during the first experiment or to achieve better selectivity and sensitivity for quantitative analysis. MS-

MS is done either by coupling multiple analyzers (of the same kind or different kinds) or with an ion trap, by doing the various experiments within the trap.

The unit of measure has become the dalton, displacing other terms such as amu. One dalton is one-twelfth of the mass of a single atom of carbon-12 (^{12}C).

Liquid Chromatography

Liquid chromatography (LC) technology gives analytical access to about 80 percent of the chemical universe that is unreachable by GC. In its simplest form, LC relies on the ability to predict and reproduce competing interactions between analytes in solution (the mobile or condensed phase) being passed over a bed of packed particles (the stationary phase). The development in recent years of columns packed with a variety of functional moieties and of solvent delivery systems able to deliver the mobile phase has enabled LC to become the analytical backbone for many industries. The abbreviation HPLC for high-pressure liquid chromatography was coined in 1970 by Csaba Horváth to indicate that high pressure is used to generate the flow required for LC in packed columns. The technique is now generally referred to as high-performance liquid chromatography, with the same abbreviation being used. HPLC is an important separation technique for the analysis of proteins and peptides because it can easily be coupled to a mass spectrometer. Moreover, the compatibility of solvents used in HPLC separations with ESI makes this hyphenated technique most commonly used in the final stage of proteomics analysis.

TIER 1: 2012 TO 2016

Currently there are two MS-based systems that may be viewed as having parameters that come close to satisfying the proposed figures of merit for the autonomous detection system using mass spectrometry. The relevant characteristics include actual system size, performance, and quantitative figures of merit, such as the probability of false-positives approaching one false-positive per year. Three significant differences between the two systems are size, the state of the sample collected, and liquid expendable.

Hamilton Sundstrand Mass Spectrometer

The modified Hamilton Sundstrand mass spectrometer (HSMS) (Basile et al., 1998; Griest et al., 2001; Hart et al., 2000) is fairly small in size, uses a liquid expendable, and collects bulk samples. The system was originally manufactured by Bruker-Franzen in the 1980–1990 time frame and then transferred to Oak Ridge National Laboratories in the late 1990s. It subsequently came into the hands of Hamilton Sundstrand. The system has matured, and the key feature is that the aerosol sample is collected in bulk in a few minutes onto a small filter paper. Microliter amounts of liquid are injected onto the spot. The liquid is the reagent tetramethylammonium hydroxide (TMAH). The spot is then heated (pyrolyzed) in a ballistic fashion to about 500–550°C within 14 seconds and maintained at that temperature for 4 minutes, and TMAH reacts with any fatty acids that are included in the contents of the spot. TMAH reacts and derivatizes the fatty acids, and fatty acid methyl esters (FAMEs) result. Fatty acids are typically found in all bacteria. There are certain fatty acids that are fairly unique to certain bacteria, but the better discriminator is the pattern of approximately 20 to 30 FAMEs that are produced to the exclusion of almost all other material in the spot. Residual char is heated to a higher temperature so as to develop a clean, fresh spot for the next cycle of aerosol collection. Meanwhile the vapors are transported into the ion trap system (MS-MS) for analysis. There is a resident database, and the system can produce an output with the likeliest bacteria present.

It is the 20 to 30 derivatized fatty acids that are the fundamental data used to describe, analyze, and identify the biological sample. It is not yet known if that is enough information to correctly identify all the agents of interest and to exclude all others at the desired probability of detection (Pd) and probability of false-positive (Pfp) levels. Very few Pd and Pfp analyses were performed by the HSMS team. There has been none published.

The HSMS has the following features:

- Vibration tested in a way that simulates the high-mobility multipurpose wheeled vehicle: HMMWV Model 1097 “Humvee.”
- Chemical ionization in addition to electron ionization. Chemical ionization significantly reduces background chemical noise (such as diesel and gasoline vapors).
- The vacuum system operates at about 35 W.

- The HSMS has four separate modules, and each module is further compartmentalized for easy troubleshooting and replacement.
- The entire HSMS is approximately 5.8 cubic feet and draws an average of 500 W power.
- Two-stage virtual impaction system to deliver bioaerosol to the heating area from 300 L/min to 1 L/min with 50–90 percent efficiency for aerosol aerodynamic diameters between 2 and 10 microns.
- Sample deposition, heating, and processing take place in a quartz tube, and the vapors are directed into the MS-MS system.
- The 330-L/min aerosol input flow rate with a 2-minute sampling time and 50 agent-containing particles per liter of air (ACPLA) equates to 33,000 particles collected, which produces S/N = 5. This is laboratory data.
- Outdoor Joint Field Trials-6 (JFT-6) at Defence Research Establishment Suffield (DRES) in Medicine Hat, Alberta, Canada, during August 2000 had *Bacillus globigii* (BG) spore and *Erwinia herbicola* aerosol releases at the 30 ACPLA level. S/N of the JFT-6 bacterial FAME spectra were between 7 and 10.
- No real quantitative figures of merit have been done with respect to false-positive (FP) and probability of false-positive (Pfp).
- The four main Category A pathogenic bacteria and numerous spore and vegetative simulants have been performed.

Bioaerosol Mass Spectrometer

The bioaerosol mass spectrometer (BAMS) produces low-end bacterial biochemical information such as basic dipicolinic acid spore information. The BAMS needs a significant reduction in size. However, the system has had a significant degree of success in real-time analysis situations.

Lawrence Livermore National Laboratory (LLNL) documented success in tackling the challenge of detection and identification of bacterial aerosols by time-of-flight mass spectrometry (TOF-MS). Much of the success is due to the multivariate data-analysis methods that delineate the simultaneously captured positive- and negative-ion mass spectra. The impetus for developing a bioaerosol-TOF-MS system originated in the modification of a system based on the analysis of ambient inorganic and

organic aerosols (Liu et al., 1997; Su et al., 2004) naturally found, generated, and released into the environment.

The bioaerosol-TOF-MS system developed at LLNL does not use chemical matrix, liquid, or solid consumables (Russell et al., 2005; Steele et al., 2003). Bioaerosol BG spores were drawn through a sizing region consisting of two lasers. A frequency-quadrupled, Q-switched Nd:YAG ablated, desorbed, and ionized each particle. Both positive and negative ions are scanned because two separate TOF-MS tubes emanate from the ion source at a separation of 180°. Typical mass spectra for a BG spore showed positive ions <150 Da and negative ions <200 Da. This was the first-ever recording of positive- and negative-ion mass spectra from the same biological particle.

The development of the BAMS (Fergenson et al., 2004) saw scrutiny on the concept of analyzing every particle in succession. This is a key point that lends itself to the autonomous detection system using mass spectrometry Pd and Pfp figures of merit. Spores of BG and *Bacillus thuringiensis* (BT) were used with no liquid reagent. The data analysis starts with positive and negative mass spectra of 350 total masses (elements). The experimental spectra are then compared to a database of organisms. If multiple standards match, then the closest spectrum match (positive and negative ions) was considered as the experimental analyte. Reproducibility depended strongly on laser wavelength and fluence. The wavelength of 266 nm was chosen because it was absorbed by dipicolinic acid (DPA) in the spores. In nature, DPA is only found in Gram-positive bacterial spores. Different bacterial growth media for both spores saw minimal mass spectral differences within each species.

Determination of individual particles was made in real time in two steps. First, a prescreening stage eliminated nonbacterial particles by analysis of the acquired spectra. The microbial-related spectra then went through mass-related criteria to refine and provide a database match to the experimental spectra. BG spores were identified 93.2 percent when compared to BT. Commonly found and commercially available white powders were separately mixed with BG and BT to test the data analysis algorithm for interference properties.

For specificity information, the spores were recognized 91 percent of the time with Gold Bond powder, 86 percent with growth media, 78 percent with Equal sweetener, 56 percent with fungal spores, and 46 percent of the time with Knox gelatin.

Single-Particle Aerosol Mass Spectrometer

The BAMS evolved into the single-particle aerosol mass spectrometer (SPAMS) system (Steele et al., 2008). The suite of biological substances originally investigated was expanded to include chemical, biological, radiological, nuclear, and explosive (CBRNE) materials as well as clandestine and illegal drug substances. The SPAMS system uses three continuous-wave laser beams to produce particle sizing properties. The SPAMS can track up to 10,000 particles per second.

The particle's position and velocity are used to predict when it passes through subsequent regions of the instrument. After the sizing region, the particle is interrogated by a laser-induced fluorescence (LIF) region to determine the presence of ultraviolet (UV) fluorescence, which indicates a biological nature. If the particle produces UV fluorescence, it is then ionized by a 266-nm laser beam. Identification of a particle occurs by mass spectral pattern matching with a database.

The tested substances produce significantly different experimental mass spectra, and no false identification or false alarms have been observed with sequential challenges of the CBRNE materials. In addition to the analytes, there is a constant background of ambient outdoor particles such that the background particles dominate or are equivalent in temporal signal responses to the particular CBRNE challenge signals.

The SPAMS system was tested at the San Francisco, California, airport. The aerosol collector and particle inlet were cleaned once per week. The ambient atmosphere internal to the airport was sampled every minute, and the spectra were recorded. Approximately 1 million particles were tracked and recorded over a 7-week period. After the recording and storage of the aerosol data, it was analyzed in the laboratory. No real-time analyses or decisions were made in the field. In any 1-minute interrogation, no more than two particles were identified as BG or pentaerythritoltetranitrate (PETN) explosive, and this resulted in zero false alarms because the 2 particles/minute was below the alarm threshold. Thus specificity is excellent.

Here are some figures of merit and comments on SPAMS:

- Disadvantages: Very large size and high cost. However, operating costs are very low.
- For SPAMS, it is essentially a single ACPLA detector and classifier with limited identification.

- SPAMS allows particles in the 0.7- to 10-micron-diameter range to pass into the system.
- Six laser beams size and track the aerosol particles.
- A second series of lasers, if necessary, produces LIF.
- Particles enter into a bipolar TOFMS that can track 50 particles/s.
- The system is autonomous and can track 10,000 particles/s up front. Most difficult when trying to detect nothing when no threat agent present! SPAMS accomplished high sensitivity and low Pfp and is very fast. After release in the lab of certain simulants in the air, it took on average of 34 s for the SPAMS to respond.

A comprehensive set of receiver operating characteristics (ROCs) curves has been performed to detail the Pfp and detection of false-negatives (Pfn) figures of merit (Gard et al., DARPA proposal, 2007 [unpublished]). To the best of our knowledge, there is no published work showing any ROC curves or Pfp and Pd figures of merit. Their ROC curves have only been presented in a DARPA proposal for the BAND program (Gard et al., DARPA proposal, 2007 [unpublished]).

The system can currently detect and identify a limited suite of samples. The time to detection is 1 minute at 1 ACPLA, and the aerosol size range is 1–10 microns in diameter. To detect 1 ACPLA in 1 minute and sampling 100 L/min with sampling efficiency of 10 percent yields 10 agent-containing particles per minute. Alarm conditions are based on the measurement and assignment of more than 1 particle.

The probability of misclassifying a particle as an agent is vanishingly small because no false-positives were observed in 5,000 BG, BT, and background particle cases. The probability of a single BT particle being wrongly identified as BG is 10^{-3}. Concerning the probability for a false alarm, the alarm conditions are based on the measurement and assignment of more than 1 particle. The probability of correctly classifying a particle as an agent can be inferred from the BG spore challenge, in which the system correctly identified the particles 93 percent of the time over BT and other background aerosols. BG remained unidentified in 7 percent of the cases. The BAMS system has a good chance at performing to and meeting most of the autonomous detection system using mass spectrometry technical requirements but with some false-negatives. This assessment can also be considered for organisms in addition to *Bacillus subtilis*. The main issues for the BAMS and SPAMS are the size and power requirements.

TIER 2: 2016–2020

Tier 2 and Tier 3 systems cannot begin to address the analytical figures of merit that are requested by DHS. Future systems just have not addressed any final or mature numbers because of the very fluid makeup and constitution of the basic research laboratory apparatus. For example, sensitivity and specificity change constantly depending on the hardware, methods, sample handling processes, and data analysis packages used. There is so much change as well as new modifications for the hardware and techniques.

Aerosol Collector Candidates

Possible candidates for aerosol collector are the Micromachined Virtual Impactor Collector and the Micromachined Radial Virtual Impactor Collector from MesoSystems Technology, Inc. However, essentially no sampling technique can ensure that the collected microbial specimen reflects the original state and can be directly used in bioanalysis (Heidelberg et al., 2000; Lee et al., 2004; Pasanen, 2001; Ren et al., 2001).

Pathogen agar plate culturing is generally the necessary step before analysis, mainly because the concentration of the collected pathogen is too low for direct bioanalysis by the methods mentioned above. Because of the size difference, the water used to rinse or wash the samplers is generally too much compared with the small amount of the pathogens collected in the samplers. Consequently, the concentration of the collected pathogens in aqueous media is too low for direct bioanalysis.

There are various possible solutions. Microfluidics, which handles liquid in the micrometer dimension, corresponding to nanoliters in volume, appears to concentrate pathogens in a relatively small amount of liquid (Baoa et al., 2008; Bhagat et al., 2011; Qi et al., 2010; You et al., 2011). Microfluidics is also economical, with much less reagent consumption, suitable for a large-scale deployment and field application (Holmes and Morgan, 2010; Li et al., 2006; Liu, 2010; Park et al., 2011). Cell capture by microfluidic chip has been reported (Jang et al., 2012; Lim et al., 2012; Loutherback et al., 2012; Reisewitz et al., 2010), but most reports focused on tissue cell capture and rarely refer to airborne bacteria cells captured directly from air.

A simple microfluidic device that is capable of fast and efficient airborne bacteria enrichment has recently been reported (Jing et al., 2013).

The initial concentration of *E. coli* bacteria suspension was 10^6 cell/mL, and an aerosol generator was used to generate the bioaerosol for 2 minutes. Under the vacuum created by a micropump, the bacteria aerosol is drawn into the channels of the microfluidic chip. At the same time, an LB culture dish is placed next to the microfluidic chip as a parallel control (sedimentation method). Bacteria may be captured through their adhering to the inside walls of the microchannels in the chip. The uncaptured bacteria will pass through the chip and enter the resuspension solution. After enrichment, 2 μL of buffer are loaded into the microchannel to wash the captured bacteria inside the microfluidic chip, and the solution is collected at the outlet for statistical analysis.

When the concentration of *E. coli* bacteria suspension was 10^5 cell/mL, there were 254 cells collected by the microfluidic chip, which is more than 4.53 times higher than the 66 cells collected by the agar-plate, direct-from-the-air sedimentation method. When the concentration of *E. coli* bacteria suspension was 10^4 cell/mL, there were still 130 bacterial particles collected by the microfluidic chip, which is 4 times higher than the 26 cells collected by the plate sedimentation method.

When the concentration of *E. coli* bacteria suspension decreased to 10^3 cells/mL, the microfluidic chip collected 56 bacteria cells, which is 55 times higher. The 130 *E. coli* bacteria captured by the microfluidic chip were enough for rapid detection methods, such as an ELISA-based test (100 bacteria are enough for ELISA and polymerase chain reaction [PCR]-based tests).

Moreover, the detection limit of the microfluidic device is much lower than that of the agar-plate sedimentation method. It can collect enough bacteria at a low aerosol concentration for a direct ELISA, loop-mediated isothermal amplification (LAMP) test, which is essential for rapid bacteria detection, especially compared with traditional bioaerosol collection techniques that need the downstream culturing or PCR amplification because of the relatively high capture limit.

Bacterial Proteome Analysis

The bacterial proteome represents the collection of functional and structural proteins that are present in the cell. The protein content of the cell represents the majority of the cell dry weight, which makes it an ideal cellular component to be utilized for bacterial characterization (Loferer-Krobacher et al., 1998).

Most of the Category A, B, and C biological threats from the Centers for Disease Control and Prevention (CDC) have their genomes fully sequenced and available for bioinformatics-based proteomics methods.

The predominant MS techniques used for bacterial identification and differentiation include ESI-MS-MS, MALDI-TOF-MS, and one- or two-dimensional sodium dodecylsulfatepolyacrylamide gel electrophoresis (1D or 2D SDS-PAGE).

MS techniques for bacterial identification and differentiation (Kollipara et al., 2011) rely on the comparison of the proteome information generated from either intact protein profiles (top-down) or the product ion mass spectra of digested peptide sequences (bottom-up) analyses (Fox et al., 2002; Pennington et al., 1997; Zhou et al., 2012). The different approaches include

- Top-down from intact proteins: Bacterial differentiation and identification are accomplished through the comparison of the MS data of intact proteins with an experimental mass spectral database (fingerprint spectrum) containing the mass spectral protein masses of the microorganisms (Demirev et al., 1999; Fenselau and Demirev 2001; Jabbour et al., 2005; Pineda et al., 2000).
- Bottom-up around 1.5 kDa: Bacterial differentiation using product ion mass spectral data of peptide sequences from the trypsin-digested proteins is accomplished through the use of search engines against publicly available sequence databases to infer identification (Williams et al., 2002).
- Middle-down: around 5 kDa (Zhou et al., 2012). This is the most mature method for the MS identification of bacterial proteins.
- Shotgun proteomics: Trypsin is used to digest and separate all peptides in an LC system for MS-MS analysis. This is the preferred method because it yields the most data.
- Peptide mass fingerprinting or peptide fingerprinting from proteins: Only for predominately expressed bacterial proteins in a MALDI-MS spectrum. Thus, either intact bacterial mass or protein extract can be trypsin-digested without any purification or separation of the proteins. This approach is usually used to target certain proteins that are overexpressed in a bacterium. Then one can compare a theoretical table of peptide masses with experimental masses; small acid-soluble proteins (SASPs) are a good example (Castanha et al., 2006; Demirev and Fenselau, 2008).

Bacterial Protein Processing

This is a critical step given that the protein portion must be isolated if MS is to be useful in the analysis of bacterial aerosols. In general, proteins isolated from lysed bacterial cells will contain constituents detrimental to their isolation, such as lipids, nucleic acids, and polysaccharides. Unfortunately, the presence of buffers, chaotropes, detergents, or cocktails of proteinase inhibitors, which are usually added to aid in protein extraction and to preserve the integrity of a proteome, may interfere with further processing and analysis of proteins. Therefore, they have to be removed from the sample before introducing the sample into a mass spectrometer (Wisniewski et al., 2009).

Jabbour et al. (2011) devised a "one-pot protein mixture purification avenue" that removes the extraneous background milieu low-molecular-weight impurities. The conventional in-solution digestion of the protein contents of bacteria is compared to a small disposable filter unit that is placed inside a centrifuge vial for the processing and digestion of bacterial proteins. Each processing stage allows the filtration of excess reactants and unwanted byproducts while retaining the proteins. Upon the addition of trypsin, the peptide mixture solution is passed through the filter while retaining the trypsin enzyme. This can be replaced with a solid-state trypsin system.

Micro-Total Analysis Systems: Raw Sample Refinement.

A micro-total analysis system (μTAS) can include bacterial background cleanup, lysis (Andersson and van den Berg, 2007), protein purification, and protein digestion where the created peptides are introduced into an HPLC column for separation. This can be thought of as a miniature "one-pot protein mixture purification avenue." A μTAS is a microfluidic device for sample processing using minimal reagents and water buffer (Dittich et al., 2006). It is characterized by a microfabricated "lab on a chip" and pressurized to allow the flow of liquid through the system. Biochips have been manufactured to include micro-LC separation columns and ESI. They can be characterized as either separate (modular) sections or monolithic (one injection-molded piece) designs. The peptide digest can be injected into a micro-LC column.

Proteomics on a Chip

Significant efforts over the past decades have been focused on the development of "proteomics on a chip" in an attempt to incorporate the various components necessary for analytical operations onto a single cost-effective platform (Feng et al., 2009; Freire and Wheeler, 2006; Henion, 2009; Huikko et al., 2003; Lion et al., 2003; Liu et al., 2006; Ma et al., 2009; Sedgwick et al., 2008; Szita et al., 2010; Tian et al., 2011; Wang et al., 2000).

Microfluidic Proteomic Reactor Performance

A comparison of the performance of the microfluidic proteomic reactor to the conventional proteomic reactor was carried out by Ethier et al. (2006). A standard protein, BSA, was processed and analyzed by a nano-HPLC-MS-MS system. All of the peptide peaks that originated from BSA were labeled on a base peak chromatogram. This result demonstrates that (1) the protein sample is digested efficiently and that (2) the proteomic reactor on a polymeric chip does not contaminate the HPLC-ESI-MS-MS with residual chemical from the polymers.

MALDI-MS

Three general processing procedures are used to generate protein ions for subsequent characterization and identification of bacteria with MALDI-MS.

The simplest uses a mixture of the bacterial sample with a matrix deposited onto a metal MALDI target (Bright et al., 2002; Demirev et al., 2001; Gantt et al., 1999; Hettick et al., 2004; Lay, 2001; Lee et al., 2002; Wang et al., 1998). In this whole-cell analysis procedure, any deliberate steps to break open or fragment the exterior cell walls are avoided in part due to logistic and procedural concerns.

The second method consists of suspending whole cells in a solvent to solubilize or extract protein species from the bacterial sample (Amado et al., 1997; Williams et al., 2003). Different types and amounts of proteins are extracted depending on the polarity of the solvent and the presence of additives (Dickinson et al., 2004). A portion of the protein extract is mixed with organic liquid matrix and analyzed by laser desorption/ionization MS. The efficiency of protein extraction was investigated

with respect to different solvents (Domin et al., 1999; Madonna et al., 2000; Ruelle et al., 2004), pH, salt content, detergent (Li et al., 1997), and other additives.

The third method uses lysis techniques (Bright et al., 2002; Demirev et al., 2001; Halden et al., 2005; Krishnamurthy et al., 1996; Lay, 2001; Owen et al., 1999; Williams et al., 2003) to deliberately break open or fragment the bacterial cell, and this allows straightforward solvent extraction of cellular proteins. A protein extract is mixed with a matrix and the mixture is analyzed by MALDI-MS.

MALDI vs. LC-ESI

MALDI-MS is used extensively in clinical and microbiological laboratories primarily for differentiation and characterization objectives for bacteria (Dworzanski and Snyder, 2005). Only single pure bacterial samples from agar-plate colonies have been performed. With ESI, mixed bacterial samples and metaproteomics (protein analysis from a mixture of many bacteria) have been performed. Overall, relatively few protein markers desorb from bacteria in MALDI. Very little detail is resident in the MALDI mass spectral signals compared to LC-ESI-ion trap MS-MS. LC-ESI yields orders of magnitude greater mass signals (Yates, 2004; Yates et al., 2009) for sophisticated data analysis techniques.

However, the number of steps needed to process bacteria is greatly reduced for MALDI compared to LC-ESI. The water expendable is not necessary for MALDI, and it is required for LC-ESI. An organic unsaturated/aromatic ring substance is essential for MALDI, while water buffer solutions are required for LC-ESI. Even if a sample is of a complex nature, both MALDI and LC-ESI benefit from a set of sample purification stages. Both necessarily require some form of sample cleanup. MALDI procedures also have used a laser impinging directly on a bacterial colony taken from a growth dish.

MALDI data analysis is based on patterns of mass spectral peaks that are compared to a database of spectral replicates. Thus the reference database must be derived from experiments. Discrimination is usually based on 10 to 20 peaks as data input (Lau et al., 2012; Liu et al., 2007). LC-ESI bacterial analysis is based on a comparison between the experimentally derived peptide/protein analysis and the protein translations from genome data banks. Discrimination is usually based on many hundreds to thousands of peaks as data input.

In terms of "bang for the buck," LC-ESI provides orders of magnitude more raw data than MALDI, and much more sophisticated data analysis and reduction packages exist for LC-ESI data than for MALDI data.

Peptide Analysis for Bacterial Identification

The peptide mixture from the "one pot" method by Jabbour et al. (2011) was analyzed by LC-MS-MS with an in-house BACid algorithm in order to compare the experimental unique peptides with a constructed proteome database of bacterial genus, species, and strain entries. The concentration of bacteria was also varied from 10^7 to 3.3×10^3 cfu/mL. The protein-processing method results in reliable identification of pure suspensions and mixtures at high and low bacterial concentrations. The peptide supernatant was concentrated and introduced into an LC system. The purpose was to provide for comprehensive processing of bacterial cell lysate proteins into a "one pot" design with no offline components, including the removal of reactants and byproducts after each step. Bacterial outer layers, membranes, and extraneous spore coat macromolecule material all need to be separated and removed from the soluble protein milieu so as not to interfere with the processing steps. Prior reagents, byproducts, and components were removed so as not to affect the protein for analyte integrity in subsequent steps. A 3-kDa molecular weight cutoff (MWCO) membrane was used without LC column or separation components; therefore, the proteins were retained during processing. Once peptides were generated on the MWCO membrane by trypsin digestion, they were passed through the membrane and loaded onto the analytical LC column for mass spectral analysis. The trypsin enzyme was retained by the membrane.

Further, different bacterial dilution protocols were performed for low-concentration studies. Dilute bacterial samples were analyzed in order to assess the peptide recovery and bacterial identification ability of the in-house bacterial classification and identification (BACid) algorithm (Deshpande et al., 2011). The "one pot" procedure and sample dilution method were investigated for identification and reproducibility concerns for a range of bacterial concentrations.

Bacterial Classification Analysis

Using the "one pot" method of Jabbour et al. (2010), analyses were performed with known and double-blind bacterial suspensions at different concentrations. Lower concentrations of bacteria in general provided a lower amount of peptide recovery. Lower bacterial concentrations of 3.3×10^3 and 3.3×10^4 organisms provided satisfactory identification capabilities. The *E. coli* K-12 strain provided a shortest single-linkage Euclidean distance in a dendrogram analysis to the database *E. coli* K-12 entry.

A double-blind sample was closest to *S. aureus* subspecies *aureus* MRSA252 at a relatively high concentration of 10^7 cfu/mL. At a lower concentration, *S. aureus* MRSA252 and *S. aureus* RF122 were determined to be the closest strains to the sample strain. The bacterium was subsequently revealed to be *S. aureus* American Type Culture Collection (ATCC) 12600. This particular strain is not contained in the database because its genome has not been sequenced. However, the analysis did include the experimental sample within the group of *S. aureus* strains.

This approach utilizes the knowledge of amino acid sequences of peptides derived from the proteolysis of proteins as a basis for reliable bacterial identification. To evaluate this approach, the tryptic digest peptides generated from double-blind biological samples containing either a single bacterium or mixture of bacteria were analyzed using LC-MS-MS. Bioinformatics tools that provide bacterial classification were used to evaluate the proteomics approach. Figure J-1 shows that bacteria in all of the double-blind samples were accurately identified with no false-positive assignment.

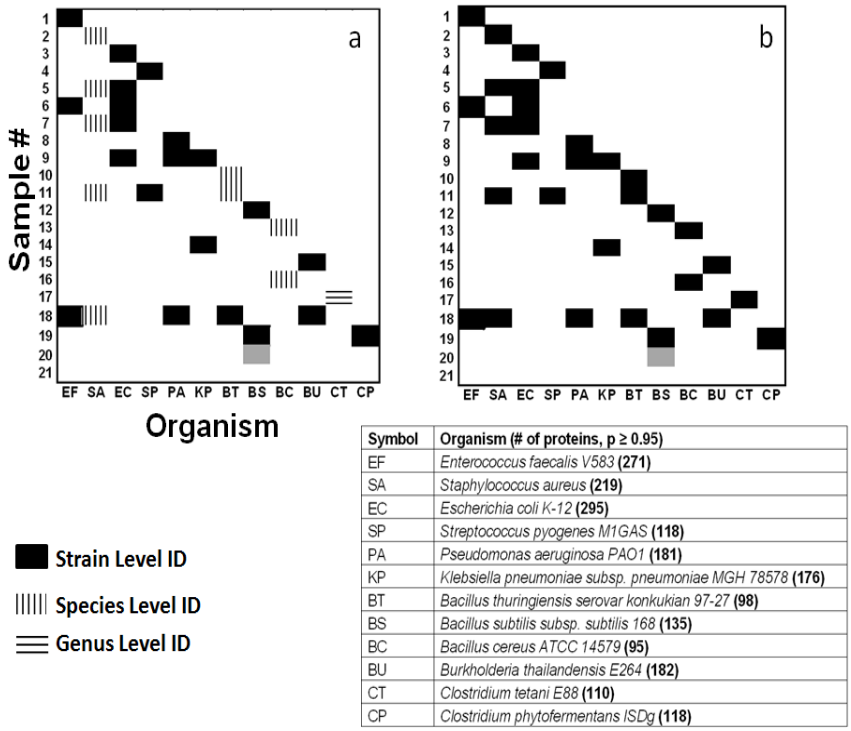
The approach also characterized double-blind bacterial samples when the experimental organism was not in the database due to its genome not having been sequenced. One experimental sample did not have its genome sequenced, and the peptide experimental record was added into the virtual bacterial proteome database. The MS proteomics approach proved capable of identifying and classifying organisms within a microbial mixture.

Several peptide searching algorithms (i.e., SEQUEST and MASCOT) have been developed to address peptide identification using proteomics databases that were generated from either fully or partially genome-sequenced organisms (Demirev and Fenselau, 2008; Ecker et al., 2005; Krishnamurthy et al., 2000). The above approach is based on SEQUEST and Agents of Biological Origin Identifier (ABOid). ABOid is based on bacterial proteins resident in an in-house proteome database translated

from an online database of sequenced microorganism genomes. The exploitation of this proteome database approach allowed for a faster search of the product ion spectra than that using genomic database searching. Also, it eliminated inconsistencies observed in publicly available protein databases caused by the utilization of nonstandardized gene-finding programs during the process of constructing the proteome database.

Blind Mixture Analysis

The BACid analysis of Sample 18 in Figure J-1 is shown in Figure J-2. BACid eliminated all the unwanted and degenerate peptides, and only the unique peptides that represented a 99 percent confidence level and above were retained for each organism. In this case, the number of



Symbol	Organism (# of proteins, p ≥ 0.95)
EF	*Enterococcus faecalis V583* **(271)**
SA	*Staphylococcus aureus* **(219)**
EC	*Escherichia coli K-12* **(295)**
SP	*Streptococcus pyogenes M1GAS* **(118)**
PA	*Pseudomonas aeruginosa PAO1* **(181)**
KP	*Klebsiella pneumoniae subsp. pneumoniae MGH 78578* **(176)**
BT	*Bacillus thuringiensis serovar konkukian 97-27* **(98)**
BS	*Bacillus subtilis subsp. subtilis 168* **(135)**
BC	*Bacillus cereus ATCC 14579* **(95)**
BU	*Burkholderia thailandensis E264* **(182)**
CT	*Clostridium tetani E88* **(110)**
CP	*Clostridium phytofermentans ISDg* **(118)**

FIGURE J-1 Accurate identification of bacteria in double-blind samples: (a) experimental and (b) actual sample key. Sample 21 is a blank. Numbers in parentheses represent the number of proteins identified. Solid box, strain level ID; vertically hatched box, species-level ID; horizontal hatched box, genus-level ID.

unique peptides varied for the different bacterial candidates. *E. faecalis* had the highest number of unique peptides, followed by *B. thuringiensis,* and *B. thailandensis* had the least number of unique peptides. Also shown in Figure J-2 for Sample 18 are six bacterial candidates near the cutoff threshold within the *Staphylococcus* genus. *Staphylococcus aureus* ATCC 3359 strain present in the blind sample has not been sequenced and has not been reported in the public domains, and thus was not part of the constructed proteome database. However, BACid was capable of providing a nearest-neighbor match to the species level (*aureus*) and thus identified the bacterium correctly as *S. aureus* subsp. aureus. It is noteworthy to mention that this bacterial strain, which is not genomically sequenced, could only be identified to the species level.

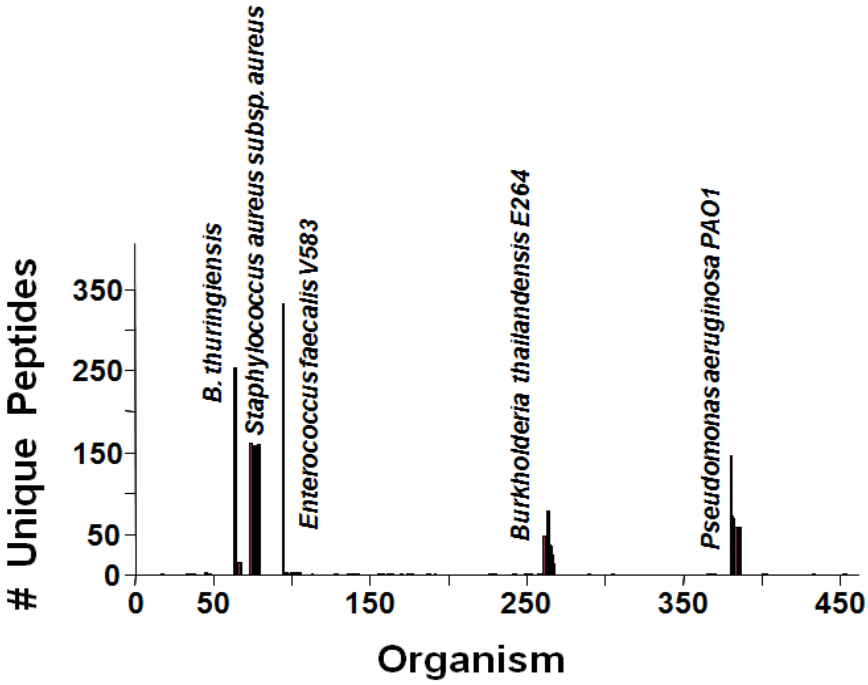


FIGURE J-2 ABOid output for LC-MS-MS data processing of Sample 18. Ordinate provides actual number of SEQUEST generated and filtered unique peptides. Abscissa represents the bacteria found at least once in the 21 experimental samples.

A significant advantage of this approach is that if a particular strain has not been sequenced and yet the species is represented in the database, it is highly likely the unsequenced sample strain will be identified to that species level. Strain-level experimental identification is indicated by a single line (see Figure J-2) in the histogram (*Enterococcus faecalis* V538) or by a grouping of lines where one line clearly dominates (e.g. *Burkholderia thailandensis* E264 and *Pseudomonas aeruginosa* PAO1) with respect to the number of unique peptides. *B. thuringiensis* has two strains resident in the database, and the two provide similar sets of peptides. This occurs because the two strains do not display peptides that clearly distinguish themselves. This blind sample was correctly identified as a mixture of five bacteria: *B. thuringiensis*, *S. aureus* subsp. aureus, *E. faecalis* V583, *B. thailandensis* E264, *and P. aeruginosa* PA01, where *S. aureus* and *B. thuringiensis* were identified to the species level, and the other three were identified to the strain level.

Genus-Level Identification

Blind sample 17 was investigated for BACid characterization. The experimental set of peptides could provide results only to the *Clostridium* genus level because all nine Clostridia bacteria (species strains) resident in the database produced a histogram (data not shown) similar to that of *Staphylococcus aureus* in Figure J-2. The experimental peptides matched that portion of the virtual proteome common to all Clostridia. Therefore, the complete experimentally derived, tryptic peptide information record was stored as a separate bacterial line item as "*Clostridium* species 1" in the database of 881 bacteria. Another aliquot of the blind sample was processed with data reduction and searching in the new hybrid database. The highest match was with the *Clostridium* species 1 entry. After the results were submitted, the identity of Sample 17 was revealed to be *Clostridium phytofermentans* ISDg. This strain does not have its genome sequenced, yet BACid was able to match the virtual proteins that are similar with the *Clostridium* genus to the experimentally observed peptides. Thus, BACid was able to characterize Sample 17 as *Clostridium* without choosing one of the nine Clostridia strains resident in the database or other bacterial genera. BACid instead matched Clostridia species 1 to the experimental peptides, which indicated that there is sufficient information in the experimental peptides to differentiate *Clostridium phytofermentans* ISDg from the nine database Clostridia strains.

The results showed that the method was effective in identifying bacteria whether the sample was composed of one organism or a mixture or even if the sample was not resident in the database. No false-positives were observed for any of the blind samples that were analyzed, including the blank sample. There are some major advantages to the proteomic method over other molecular biology methods, such as the DNA-based methods, in that (a) no prior information about the sample is required for analysis, (b) no specific reagents are needed in the analysis process, (c) proteomics MS is capable of identifying an organism when a primer/probe set is not available, (d) proteomics MS requires less rigorous sample preparation than PCR, and (e) proteomics MS can provide a presumptive identification of a true unknown organism by mapping its phylogenetic relationship with other, known pathogens.

Sensitivity Performance of Mass Spectrometry–Based Proteomics

The MS proteomics method has shown promising results in specificity and sensitivity. While the latter parameter is highly dependent on the MS physical limit of detection, enhancing the biological sample processing is a crucial step to ease such dependency. Table J-1 shows bacteria and their sensitivity limits for the MS proteomics method.

TIER 3: BEYOND 2020

Future systems may combine various techniques, such as PCR with LC-ESI-MS-MS or MALDI-MS-MS or ESI-TOF-MS or antigen-antibody with LC-ESI-MS-MS or MALDI-MS-MS or ESI-TOF-MS.

The PLEX-ID is a product (Havlicek et al., 2013) that, although recently introduced (Jacob et al., 2012), has had a short-lived life in the commercial market. It and its predecessor, the Ibis T5000, were developed by Ibis Biosciences, Inc., which was acquired in 2009 by a subsidiary of the Abbott Diagnostics Group (Abbott Molecular Inc.). The product featured nucleic acid amplification (PCR) coupled with ESI-TOF-MS to carry out base-composition analysis (Ecker et al., 2008). The PLEX-ID instrument received the CE marking in March 2012 along with three assays for use on the system: PLEX-ID Viral IC Spectrum, PLEX-ID BAC Spectrum BC, and PLEX-ID Flu (Ibis, n.d.). In 2011 the company

TABLE J-1 Sensitivity Limits of Mass Spectrometry Proteomics Method

		Reproducibility N=8		Specificity	Blind
Biological Agent Tested	**Analytical Sensitivity**	**% Detected**	**CV (95% confidence ID)**	**Positive ID**	**% detected**
Yersinia pestis CO92	1.0^4 cfu	100	5%	√	100
B. anthracis Ames, Sterne	6,000 cfu	100	5%	√	100
Burkholderia mallei/pseudomallei	2,000 cfu	100	4.2%	√	100
C. burnetii NMQ	8,000 cfu	90	5%	√	100
F. tularensis type-A/type-B	1.1×10^4 cfu	100	5%	√	100
E. coli O157/O104/O111 and O26	1,000 cfu	100	2%	√	100
Dengue virus	1,200 cpu	100	12%	√	88
Vaccinia	850	100	8%	√	92
Ricin toxin	75 pg	100	11%	√	95
SEB toxin	25 pg	100	9%	√	95

also introduced the PLEX-ID Biothreat Assay. The system was reported to enable the identification and quantification of a broad set of pathogens, including bacteria (Sampath et al., 2012), all major groups of pathogenic fungi (Kaleta et al., 2011), protozoa, and the major families of viruses (MacInnes et al., 2011). In September 2012 Abbott discontinued the production of the PLEX-ID system; however, it did not exclude the possibility of developing a smaller, cheaper, and faster device based on similar principles in the future.

There are many research reports on viral typing performed by MALDI (Gijavanekar et al., 2012) or ESI (Jeng et al., 2012). There is a market gap in this field, although there are speculations about the renaissance of commercial techniques based on nucleic acid sequencing (Jeng et al., 2012) because the past Abbott PLEX-ID product was discontinued in September 2012.

Memory effect has not been found for the lab-made devices (Wu et al., 2004). After each digestion, the substrate and products left in the microchannels were cleaned out by pumping fresh water through the microchannels for a few minutes. Subsequently, a blank solution of 2-mM NH_4HCO_3 (pH 8.0) buffer was allowed to flow into the microchannels as for sample digestion, then collected and checked for any remaining samples in the channels using MALDI-TOF-MS detection procedures. No detectable peptide fragments were found after using the cleaning procedures described above, which indicates that the micro-reactors are less susceptible to memory effect. The lab-made micro-reactor devices can be used at least 50 times in one week without noticeable loss of activity with a proper storage at 4°C; the two ends of the microchannel were sealed to avoid drying-induced enzyme degradation (Shi et al., 1999). In fact, the ability to alter the digestion time by varying the flow rate could provide a powerful means to achieve the desired extent of digestion or to compensate for enzyme activity loss.

Finally, a new MS system currently being developed by Torion could be either Tier 2 or Tier 3. Torion has introduced a portable, ruggedized 32-pound, battery-operated GC-MS capable of providing two to three hours of operation on a single battery. The next-generation system is proposed to be on the order of 1,200 cubic inches, 15 pounds, and 40 watts of power.

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