# Rapid Sensors for Biological-Agent Identification

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We have developed genetically engineered white-blood cells and inexpensive sensor hardware for the rapid identification of pathogens and toxins. The assays we have developed by using these cells demonstrate the best known combination of speed and sensitivity. In addition to detecting pathogens, CANARY (for Cellular Analysis and Notification of Antigen Risks and Yields) detects soluble protein toxins—an important class of potential bioweapon—and DNA and RNA sequences. CANARY's capabilities open possible applications in pathogen genotyping, virulence testing, antibiotic resistance screening, and viability assessment. For biological defense applications, we have incorporated CANARY technology into a flexible biological-aerosol sensor platform called PANTHER that can form the core of a family of mission-specific bio-aerosol identification sensors useful as standalone sensors for site/building protection, emergency response, rapid screening, and environmental monitoring.

» **The growing threat of biological agents,** new diseases, and food-borne pathogens has created an increasing need for rapid and sensitive detection before the target population becomes infected. The incident of the anthrax letters in October 2001, the emergence of Severe Acute Respiratory Syndrome (SARS) and the West Nile virus, and repeated occurrences of food-borne pathogens illustrate how necessary such detection is. Detect-to-protect or detect-to-warn operation is generally possible only with an instrument that can detect and identify the pathogens in a few minutes or less. Merely detecting the presence of biological particles (as bioparticle triggers do—see "Advanced Trigger Development" by Thomas Jeys and colleagues, page 29) is insufficient, as there are generally many kinds of benign bacteria and other organisms routinely present in the air and often in or on the water and food that we consume. Detect-to-protect operation therefore requires not only that the particles be detected but also that they be identified, all within a short time (Figure 1). Applications that could benefit from CANARY (for Cellular Analysis and Notification of Antigen Risks and Yields) technology include biological aerosol sampling, point-of-care diagnostics, pre-symptomatic diagnosis in the aftermath of a biowarfare attack, detection of agricultural pathogens at ports of entry, or screening of perishable food supplies.

Such rapid diagnostic tests could be applied in a variety of settings. Medical care workers would prefer to be able to diagnose patients within a few minutes at the point of care (e.g., doctor's office, hospital bedside) rather than have to submit samples for testing at remote facilities where the results often take several days to obtain. In addition, rapid identification of plant and animal dis-

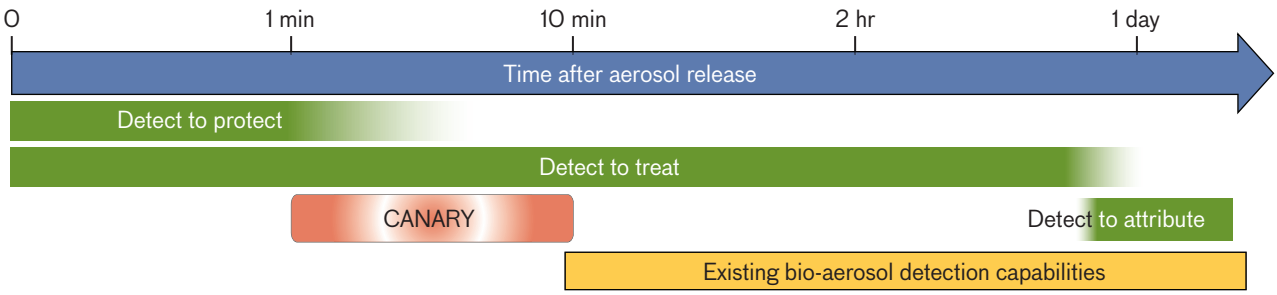


**FIGURE 1.** CANARY's (Cellular Analysis and Notification of Antigen Risks and Yields) rapid response uniquely enables coverage into the detect-to-protect region for an aerosol release.

eases will help stop the spread not only of emerging nonindigenous plant pathogens (e.g., citrus canker) but also animal pathogens of significant economic impact (e.g., foot-and-mouth disease). Agricultural inspectors or veterinarians could use such rapid tools at customs portals, fieldside, or penside to make quick determinations and thereby prevent the further spread of infections.

## CANARY Bioelectronic Sensor

Against these threats and to solve the limitations of the existing technologies, we have developed an array of assays and instruments that provide rapid (1 to 5 min) identification of pathogens in air, food, medical samples, and plant tissues. Our novel, biologically inspired technology and associated sensors demonstrate the best combination of speed and sensitivity yet demonstrated for pathogen identification.

Our technology is based on genetically engineered B cells, a type of white blood cell that binds to and recognizes pathogens quickly and assists other parts of the immune system to fight the infection. B cells are the fastest pathogen identifiers known (intrinsic response in <1 second). We have modified them to bind specifically to the pathogens of interest and within seconds emit photons to report that the binding event has occurred. Two routine genetic modifications enable engineered B-cell lines to express cytosolic aequorin, a calcium-sensitive bioluminescent protein, as well as membrane-bound antibodies specific for pathogens of interest [1, 2]. The crosslinking of membrane-bound antibodies by a polyvalent antigen induces a signal-transduction cascade that sequentially involves tyrosine kinases, phospholipase C, and inositol triphosphate ($IP_3$). $IP_3$ activates calcium channels, thereby increasing cytosolic calcium from both internal stores and the extracellular medium [3], which activates the aequorin, causing it to emit light [4, 5].

This sensor concept, shown in Figure 2, which we call CANARY, can detect <50 colony-forming units (cfu) of pathogen in less than 3 minutes, including the time required to concentrate the samples [6]. In contrast, even state-of-the-art immunoassays take at least 15 minutes and have a much higher limit of detection [7]. While the polymerase chain reaction (PCR) can be both highly specific and sensitive, most reports cite protocols that take longer than 30 minutes. An ultrafast PCR with detection of 5 cfu in only 9 minutes has been reported [8]. However, even when coupled with the most rapid sample-preparation technology the total assay requires 20 to 30 minutes to complete [9, 10]. Because of its unique combination of speed and sensitivity, CANARY has important benefits for pathogen identification in medical diagnostics, biowarfare defense, and other applications.

We have developed a genetic-engineering system that allows efficient production of B-cell lines that react specifically and rapidly to a variety of pathogens. Antibody genes cloned from hybridomas (cell lines that produce a single monoclonal antibody) are inserted into expression vectors. These vectors are transfected into a parental B-cell line that expresses active aequorin, and the cells are screened for their response to pathogen. These genetically engineered CANARY cells can be used separately in a single identification assay, or as many as three can be combined to achieve a multiplexed assay. Alternatively, several antibodies can be expressed in a single cell line to provide a classification assay. It is also feasible to create B cells that emit at different wavelengths of light, enabling multiplexed assays that simultaneously distinguish among several targets.

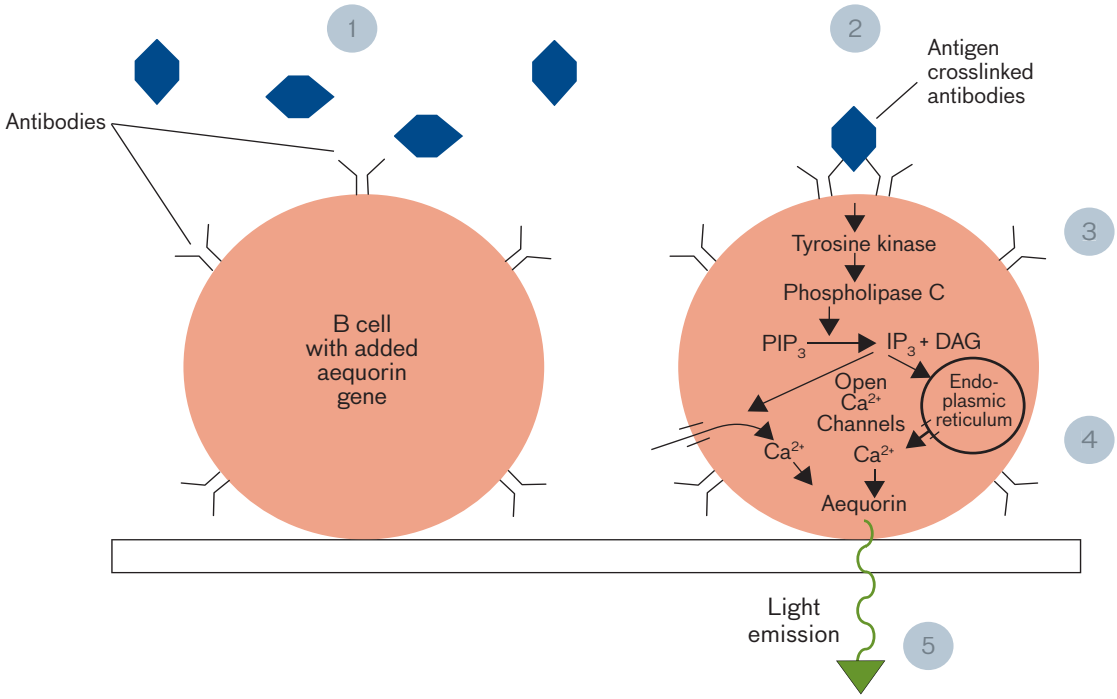


**FIGURE 2.** The fundamental principles of the CANARY bioelectronic sensor include genetically engineered B cells that emit photons in response to specific bio-agents, and a photodetector that measures the luminescence. (1) B cells are exposed to bio-agents in test sample, (2) B-cell antibodies bind to bio-agents, (3) the biochemical signal amplification enters its final stage (tyrosine kinase to $IP_3$ + DAG) and releases $Ca^{2+}$, (4) $Ca^{2+}$ makes aequorin emit photons, and (5) photons are detected from cells.

The generation of genetically engineered CANARY cells requires several months, but we have also developed a CANARY cell line whose specificity can be engineered in days. This approach takes advantage of a receptor that binds the constant region of antibodies, leaving the antigen-binding region of the antibody free. After binding bacteria with the captured antibodies, the receptor initiates a signal cascade, similar to the one induced by the crosslinking of membrane-bound antibodies on B cells, that activates aequorin. We have demonstrated the development of a rapidly engineered CANARY cell that, though not as sensitive as the genetically engineered B cells, requires days instead of months to produce.

We have also developed sensors and assay methods that present the suspect materials to B cells and acquire the optical identification signals. The CANARY assay performed in these sensors involves a centrifugal format that provides excellent speed and sensitivity for the detection of bacteria and large viruses. When diffusion controls the interaction between B cells and pathogen, the signal is slow and almost indistinguishable from background, indicative of the low rate of interaction. Figure 3 compares the results obtained with diffusion (0 sec pre-spin/0 sec spin) to those obtained with centrifugal concentration. When the agent and cells are mixed together, then concentrated by centrifugation for 5 sec, the signal is improved and the response faster (0 sec pre-spin/5 sec spin). However, optimal results are observed when the slower-sedimenting agent, *Francisella tularensis* in this example, is centrifuged prior to the addition of the cells (60 sec pre-spin/5 sec spin). This format ensures that a large number of cells come into physical contact with antigen within a short time, thereby providing a major improvement in sensitivity and speed. After additional improvement of the assay protocol, we can now detect as little as 60 (cfu) of *F. tularensis* in less than three minutes, including the time it takes to preconcentrate the agent. Furthermore, the CANARY sensor exhibits a wide range of sensitivity, detecting concentrations ranging over seven orders of magnitude, as shown in Figure 4.

This excellent combination of speed and sensitivity can also be demonstrated with cell lines expressing an antibody specific for the F1 antigen of *Yersinia pestis* (*Yp*), shown in Figure 4. When concentrated in the

# Pathogen Identification Technologies

Prior to CANARY, the existing technologies were simply too slow and bulky for rapid response to biowarfare attack.

**None of the existing pathogen-identification technologies** have been fast enough to provide a detect-to-protect capability, beginning with the first means for scientific identification of biological pathogens, the culture-plate approach used by Koch, Pasteur, and Petri in the nineteenth century (Figure A) [a, b]. While culturing is useful for isolating and multiplying certain bacteria, this method typically takes several days, and is therefore much too slow for the subject application.

In the 1950s, workers began to develop tests called immunoassays that use antibodies (physical binding, or recognition, proteins that are made by B cells, a type of white blood cell, in the bodies of vertebrates) to bind to pathogens, as shown in Figure B [b, c]. While this process can be reasonably sensitive (detection of a few thousand particles), it is generally too slow. It requires about 15 minutes for the binding events and detection to occur. A home pregnancy test is one example of this technology.

Antibody assays typically examine the surface features of pathogens; other assays test for the presence of genetic material. One such technique is the polymerase chain reaction (PCR). PCR uses natural enzymes and synthetic fluorescent DNA tags in a heat-cycling process to copy specific target (pathogen) DNA sequences. Each heat cycle results in a doubling of the amount of target DNA present, and an increase in fluorescent signal. After a number of cycles, the fluorescent signal produced during the doubling process reaches the detection threshold (Figure C), and the sample is declared positive. In the absence of pathogen, no fluorescence is produced and this threshold is not achieved. While considered the gold-standard laboratory test for specificity (since it detects the genetic code for building organisms, including pathogens), DNA detection by PCR is still a relatively slow process, taking between 20 minutes and 2 hours. [d, e].

**FIGURE A.** ***Bacillus anthracis*** **colonies appear on a culture plate after several days of incubation. The quantitative measurement of the colonies adds to the already elongated analysis time.**

Another class of pathogen identifiers that beginning in the 1980s has reached some prominence in the biology community is mass spectrometry [f], shown in Figure D. The suspect pathogens are placed at one end of a vacuum chamber and are blasted by a laser or charged-particle beam that liberates protein and amino-acid components of the pathogens from their surface. These components then fly down the chamber and are sorted and detected according to mass and charge.

Mass spectrometry can be made relatively fast (2 to 5 min-

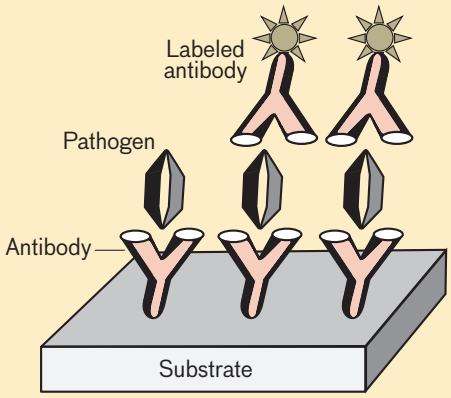


**FIGURE B. The immunoassay technique employs antibodies on a substrate that bind to pathogens. If the binding sites on the antibodies match the corresponding surface features on the pathogens, the pathogens will adhere to the antibodies. Corresponding labeled antibodies wash over the surface, interact with the bound pathogens, and signal the detection, typically by changing color.**

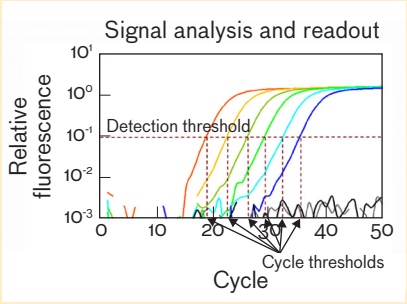


**FIGURE C.** **Polymerase chain reaction (PCR) doubles the concentration each cycle. The concentrations shown by the gray and brown curves never reach the detection threshold, while the rainbow-colored curves depict DNA that is detected above threshold at various cycles.**

utes, and some incubation of the pathogens with an acid are required before the beam is turned on). Given typical mixes of environmentally occurring biological particles (and background contaminants), however, it does not provide enough specificity to do true identification. In addition, the instrumentation is typically bulky and heavy (about 200 lb), is difficult to miniaturize while retaining mass selection, and requires a highly skilled level of operation and maintenance. Hybrid approaches combining mass spectrometry with PCR have been developed that have advantages for identifying so-called unknown unknowns in a laboratory setting. However, they require 2000 lb high-mass-resolution spectrometers, and the assays take several hours to complete [g].

Bio-aerosol analysis has, in the past, been achieved by combining wetted-wall cyclone air collectors, which collect airborne particles into a fluid, with a form of immunoassay. Such systems usually collect air for about 5 minutes by using a large and high-power-consumption fan, whereupon the collection fluid is passed via a series of tubes and valves to the immunoassay strips. These strips are read optoelectronically at the end of their 15 min incubation. In total these instruments take about twenty minutes to detect and identify bio-aerosols, which is too long for detect-to-protect operation. Another popular method is to simply collect aerosol particles onto dry filters for roughly 12 hours and then take them to a laboratory, where they are processed for pathogen analysis. In common practice this method yields results in about 36 hours, providing only a detect-to-treat capability.


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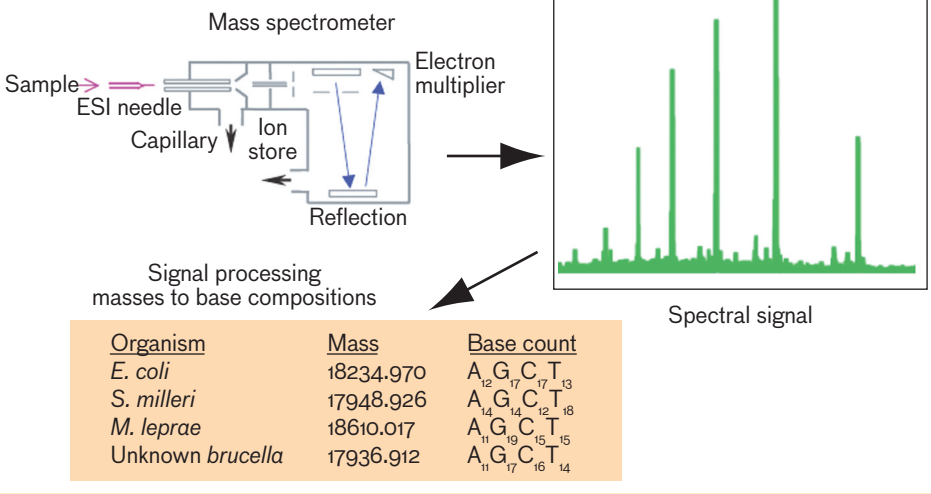


**FIGURE D.** **The mass spectrometer splits pathogens into their protein and amino-acid components. Analyzing the spectral intensities of the ions gives quite accurate mass values, which can be interpreted and mapped to existing microbes. ESI stands for electrospray ionization.**

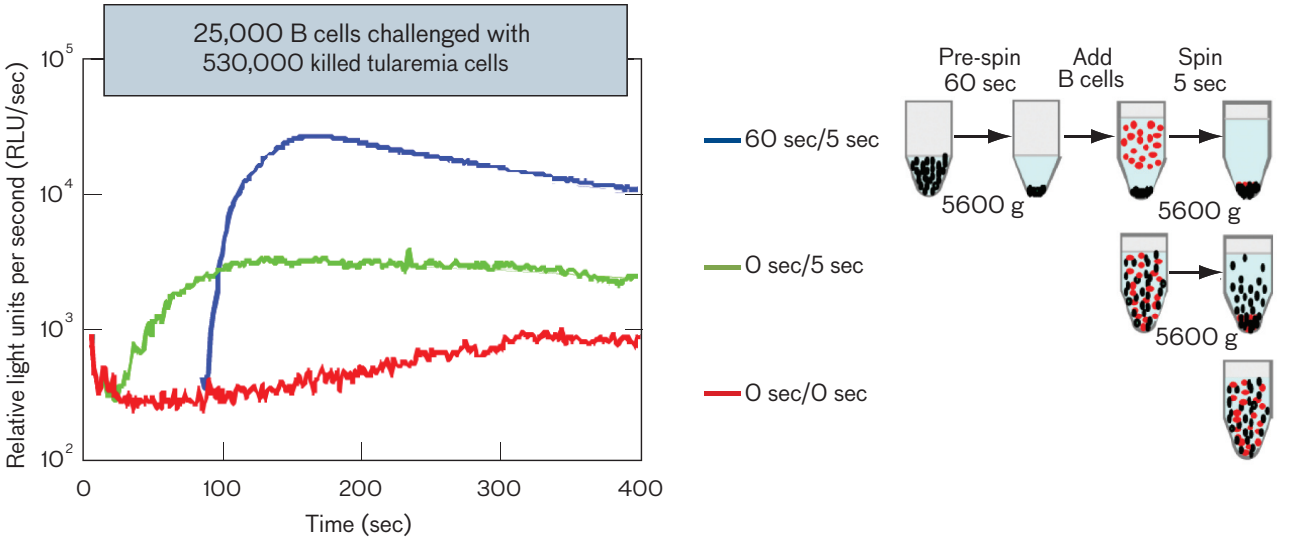


**FIGURE 3.** Centrifuging increases the speed and sensitivity of the CANARY assay. The graph demonstrates the efficacy of preconcentrating the agent and using centrifugation to deliver the B cells, as compared to using a single centrifugation or noncentrifugal method.

centrifuge luminometer, as little as 45 cfu of formalin-inactivated *Yp* are detected, but there is no response to relatively large numbers of *F. tularensis*. In fact, for most pathogens we have tested that are large enough to be concentrated in a microcentrifuge, we have observed similar levels of sensitivity, approximately 50 cfu or plaque-forming units (pfu). However, the limit of detection (LOD) is in the region of 50,000 pfu for pathogens such as viruses that are too small to be quickly sedimented in a microcentrifuge, although slightly longer centrifugations can increase the sensitivity as much as a hundredfold. When the sensitivity of the *Yp*-specific cell line was tested repeatedly over several months, the CANARY sensor could detect 20 cfu 62% of the time, 50 cfu 79% of the time, 200 and 2000 cfu greater than 98% of the time, and 20,000 cfu 100% of the time. A false-positive rate of only 0.4% (total number of tests is $n = 1288$), combined with a level of sensitivity approaching that of PCR and an assay that can be performed in a few minutes, makes CANARY one of the most promising pathogen-identification technologies currently in development.

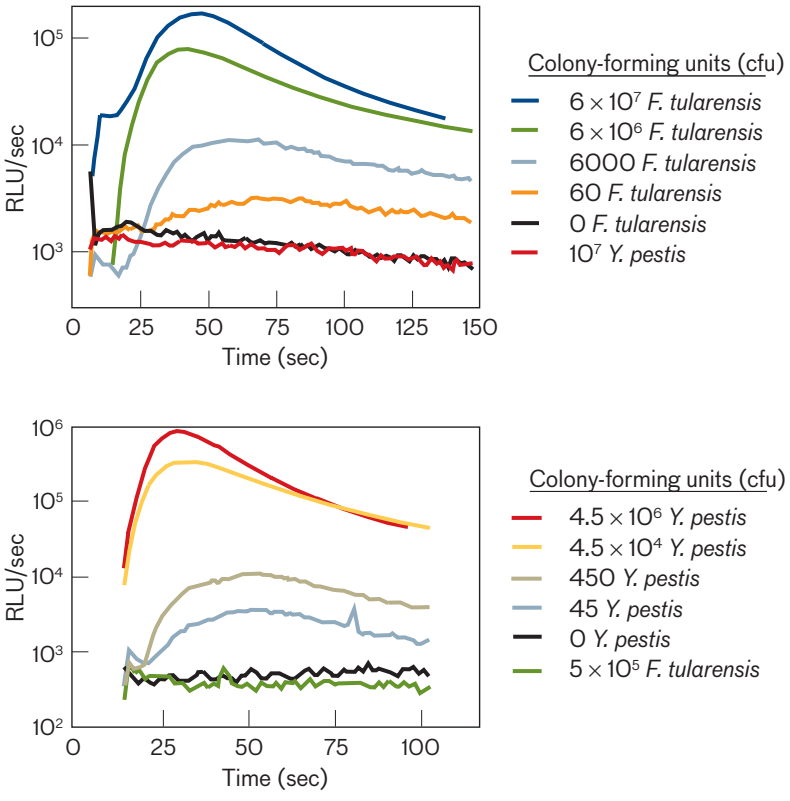


**FIGURE 4.** The dose-response curves for inactivated *Francisella tularensis* (top) and *Yersinia pestis* (bottom) show that the cells respond very rapidly over a wide dynamic range of agent concentrations. Additionally, the specificity of the system is demonstrated by the lack of cross-reactivity to other pathogens. The entire manual assay, including precentrifugation of the agent, takes less than three minutes.

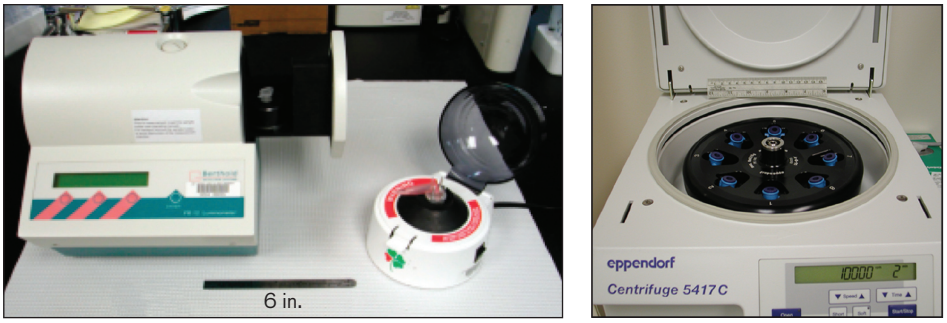


**FIGURE 5.** The single-channel CANARY sensor, shown on the left, consists of two commercial-off-the-shelf (COTS) components: a Berthold Detection Systems FB12 luminometer and a VWR minicentrifuge. An optional Eppendorf 5417C swing-bucket microcentrifuge (right) enables parallel sample preprocessing for increased throughput.

## Sensor Development

Improving single-channel hardware capable of performing optimal CANARY assays was the first hardware development task for this program. We pursued two parallel paths to achieve this goal: (1) developing of custom design concepts for a single unit capable of spinning and analyzing the CANARY samples, and (2) examining commercial-off-the-shelf (COTS) luminometers and minicentrifuges that could be modified, or preferably used without modification, to perform single CANARY assays. The outcome of that process was the identification of inexpensive COTS hardware that improved CANARY assay procedures and performance. The optimum hardware combination, shown in Figure 5, consisted of the Berthold Detection Systems FB12 luminometer used in conjunction with a VWR International minicentrifuge fitted with a custom rotor to enable spinning of up to four CANARY samples in the optimum configuration.

The procedure for using the single-channel sensor begins with an approximately two-minute pre-spin at >6000 relative centrifugal force in a conventional swing-bucket microcentrifuge, if available, or in the VWR minicentrifuge. We add a drop of B cells to the sample and spin it for 5 seconds. There is sufficient time before the signal peaks to transfer the sample to the luminometer for signal readout and CANARY identification. With this hardware configuration and parallel sample pre-spins, a single user can process up to 25 samples per hour.

## Sixteen-Channel Sensor Development

In its most sensitive form, a CANARY measurement consists of preparing a sample in a transparent tube, introducing an aliquot of specially prepared B cells into the tube, driving the B cells to the bottom of the tube by using a quick centrifugal spin, and measuring the light output from the tube with a photon-counting sensor. In the laboratory, most CANARY measurements have been made sequentially, one sample at a time. For a higher throughput 16 channel sensor, we have designed and built a rotary format that integrates centrifugation and photomultiplier tube (PMT) readout into one small package, shown in Figure 6. This unit can spin and read sixteen samples simultaneously, and can process roughly one hundred samples/hour. Additionally, we ensured that power consumption was low enough to enable inclusion of a battery into the enclosure for battery-powered operation. We accomplished these goals by building the sensor components into a small COTS transportation case that was water-tight and light-tight, and by using a smaller motor and controller that was capable of spinning the rotor by using a 24 V DC power source. The laptop that operates the portable 16 channel sensor runs custom software written to be compatible with

**FIGURE 6.** The portable 16 channel CANARY sensor is built into a light-tight and water-tight case and contains provision for conversion to battery-powered operation. The laptop is used to operate the sensor with custom software written to simplify collection and organization of sample and reagent information, as well as perform automated identification based on signal characteristics.

Microsoft Windows. The software incorporates multiple user-defined fields for tracking sample and reagent details and storing them along with the resulting data. In addition, a computer algorithm was written that analyzes the individual raw-data signals and displays an alarm when the signal characteristics are consistent with the presence of a specific pathogen.

The unique combination of sensitivity and speed provided by CANARY has many applications, including the screening of food supplies and as a medical diagnostic technique. Therefore, we tested the ability of CANARY to perform with several complex matrices such as produce, meat, and clinical samples. Table 1 shows the limits of detection for various agents in complex matrices. Pathogenic *Escherichia coli* O157:H7 is one cause of food-borne illness and has been found to contaminate spinach, lettuce, cider, ground beef, and cantaloupe. We developed a B-cell line that recognizes *E. coli* O157:H7, as well as an assay to detect its presence on fruits and vegetables. Lettuce (25 g) was artificially contaminated with inactivated *E. coli* O157:H7 and shaken in sterile bags with extraction medium. The supernatant was transferred to a syringe and passed through a 5 μm filter to remove large particulates. Then we centrifuged the eluate to concentrate the bacteria, and replaced the liquid with assay medium. Cells responsive to *E. coli* O157:H7 were added to the sample and assayed in the centrifuge luminometer format. With this simple, rapid sample preparation we are able to demonstrate detection of as little as 500 cfu/g in 5 minutes or less. These results compare favorably with several reports that used PCR to detect bacteria in food, giving a limit of detection of 10 to 10,000 cfu/g or mL.

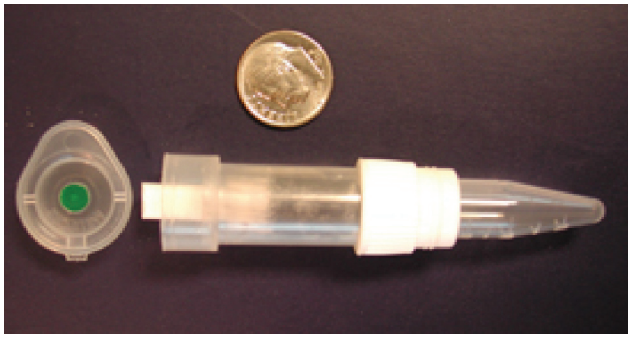

**FIGURE 7.** A centrifugal filter device consists of a 5 μm basket filter (left portion), and a detachable assay tube (right portion), shown here with a dirty sample swab inserted in the filter basket.

### Human Samples

We have developed a centrifugal filter device, shown in Figure 7, that allows for efficient and rapid elution of bacteria from swabs that have been used to wipe the surface of complex matrices. The 5 μm filter retains large particulates that interfere with the CANARY assay, while allowing the bacteria to pass through. Centrifugal force both facilitates elution of the swab and concentrates the

**Table 1: Limit of Detection of the CANARY Assay**

| MATRIX | AGENT | LOD |
|---|---|---|
| Lettuce, apple, pineapple | *E. coli* | 500 cfu/g |
| Chicken | *E. coli* | 70 cfu/g |
| Nasal swabs | *B. anthracis* spore | 50 cfu |
| Urine | *C. trachomatis* | 500 EB* |
| Whole blood | *Y. pestis* | 1000 cfu/mL |
| Urine, whole blood | *Botulinum* toxin A | 16 ng/mL |
| DFU* filter eluates | *B. anthracis*, *Y. pestis*, *F. tularensis*, *vaccinia* virus | $10^3 - 5 \times 10^4$/mL |
| Geranium extract | *R. solanacearum* | 100 cfu/mL |
| Potato extract | potyvirus | 50 ng/mL |

* EB stands for elementary bodies. DFU stands for dry-filter unit.

bacteria at the bottom of the assay tube, ensuring good sensitivity during the identification step. This device has been used to sample and process both nasal swabs and the surface contamination of chicken. In the case of nasal swabs we have identified as few as 50 cfu of *Bacillus anthracis* (*Ba*) spores added to nasal swabs prior to sample preparation. Because the total assay time is less than five minutes, CANARY could provide an excellent first screen for people who may have been exposed to aerosolized *Ba* spores and thereby allow immediate treatment.

There is also a need for rapid point-of-care diagnostic tests to ensure treatment and control of diseases, such as those which are sexually transmitted, for which there is a high rate of medication noncompliance. *Chlamydia trachomatis* (*Ct*) is a sexually transmitted disease that has a high prevalence, can cause pelvic inflammatory disease and fertility problems, and is underdiagnosed because of the high number of asymptomatic cases [11]. Historically, the disease has been diagnosed by using cervical or urethral smears with tests that require considerable time and expertise. Although the elementary bodies (EB) of the organism can be found in the urine, a less invasive sample to collect, they are present in such low numbers that until now the only tests sensitive enough to be effective are those which amplify nucleic acids. According to a recent report, the concentration of *Ct* in urine from infected patients was determined to range from 30 to $2 \times 10^5$ EB/mL [12] by using a quantitative ligase chain reaction, an assay that takes several hours to perform. The procedure for preparing a urine sample for the CANARY assay is simple. The sample is passed through a 5 $\mu$m syringe filter, 0.5 mL aliquots are centrifuged for two minutes into a pellet, the supernatant is decanted, the pellet is resuspended in 0.5 mL of assay medium, and the sample is centrifuged again for two minutes. We were able to detect 500 *Ct* EB in urine in less than five minutes, which demonstrates that CANARY, utilizing a non-invasively obtained test sample, would be useful as a rapid, sensitive assay diagnosing of *Ct* infections.

Whole blood is a difficult matrix to assay because of its opacity and because it contains both activators and inhibitors of the CANARY assay. The method we have developed relies on plasma-separation tubes (PST) and differential centrifugation. This process uses a thixotropic gel with a density between that of plasma and blood cells. The gel forms a barrier between the plasma and cells during centrifugation. Any bacteria or viruses present in the blood remain in the plasma phase after centrifugation, and can then be harvested and tested in CANARY. Figure 8 shows a prototype device assembled from COTS parts that enables the separation of whole-blood samples in three rapid, simple steps. The total time required from blood collection to pathogen detection is approximately five minutes. With the PST method, the LOD is approximately 1000 cfu of live, avirulent *Yp*/mL whole blood, or 125 cfu per CANARY assay.

## Plant Pathogens

The United States Department of Agriculture Animal and Plant Health Inspection Service Plant Protection and Quarantine Center for Plant Health Science and Technology sponsored a program for Lincoln Laboratory to develop the CANARY sensor for detection of plant pathogens, with the eventual goal of implementing CANARY in U.S. Department of Agriculture diagnostic laboratories and as detectors at ports of entry into the United States. Over the course of this project, CANARY B cells specific for three plant pathogens—a bacterium (ralstonia), a virus (potyvirus), and a fungus (phytophthora)—were developed, along with sample-preparation protocols for the tissue infected by these pathogens. Since one of the key

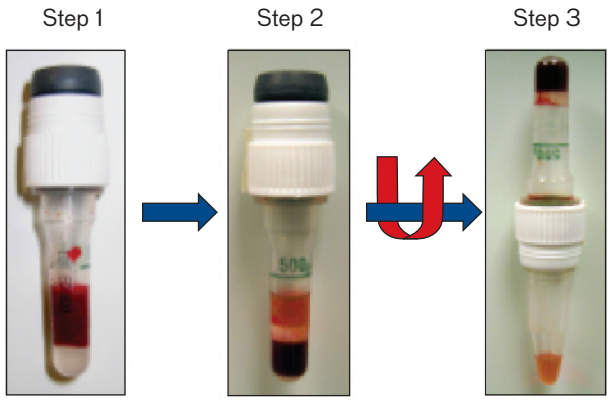


**FIGURE 8.** Whole-blood processing, with this device assembled from COTS parts, takes the following steps: (1) collect 0.5 mL whole blood into heparinized plasma-separation tubes (PST), (2) centrifuge the tube at 3500 g for 90 sec, and (3) replace the stopper with threaded CANARY assay tube and invert the device to collect plasma into assay tube. The PST and connector are removed and discarded. The plasma is diluted with the assay medium, centrifuged to pelletize the pathogen, and tested with pathogen-specific CANARY cells.

features of CANARY is the speed of the assay, we focused on developing sample-preparation techniques that would not significantly increase the overall time of the assay, yet allow for efficient extraction of the pathogens from various plant tissues.

### Potyvirus Detection

There were several unique challenges involved with preparing plant tissue to yield a sample compatible with the CANARY assay yet still allow rapid, sensitive detection of potyviruses. Potyvirus is a small virus, and although CANARY can detect small viruses, the sensitivity of the assay is usually less than what is attainable with pathogens that can be readily sedimented by low-speed centrifugation (i.e., the standard CANARY assay). Additionally, the antibodies expressed on the B cells recognize a cryptotope (an epitope found not on the virion surface but rather on coat-protein subunits found within the intact virion) that is inaccessible to binding by the B-cell antibody. Finally, the plant tissues must be ground to liberate the intracellular virus, a process that creates particulate matter that can interfere with the assay. All of these issues were overcome by capturing the potyvirus on polystyrene beads. The binding of the virus to the polystyrene bead causes the virus coat protein to unwind enough to expose the cryptotope and allow binding to the antibodies expressed on the B cell. The bead-bound virus can be easily pelleted by low-speed centrifugation to greatly increase the sensitivity of the assay. If magnetic beads are used, the virus can easily be purified from plant debris. Since the binding of the virus to the beads occurs almost instantaneously, the entire assay takes approximately 5 minutes from tissue collection to virus detection.

### Phytophthora Detection

Although phytophthora is large enough to be sedimented by low-speed centrifugation, the plant debris generated by macerating the plant tissue to liberate the organism co-sediments interferes with the assay. To circumvent this problem, we capture the mycelia with magnetic beads coated with a second phytophthora-specific antibody (i.e., an antibody that recognizes a different epitope from the antibody expressed on the surface of the B cell), allowing the pathogen to be pulled away from the debris. Using a magnetic pick-pen, we can easily transfer the bead-bound phytophthora to an assay tube, and the CANARY assay can then be performed. We were able to demonstrate a dose-dependent response to both live *Phytophthora infestans* and *P. capsici mycelia*, as well as detection of *P. infestans* in seeded potato tuber extract.

### Toxin Detection

CANARY has demonstrated an exceptional ability to detect and identify bacteria and viruses because each organism has on its surface many copies of a specific antibody binding site, as shown in Figure 9. Protein toxins (such as ricin, botulinum neurotoxin, or SEB) have only a single copy of any given antibody binding site. This means that the antibodies on the surface of the CANARY cell will not be crosslinked by soluble monomeric toxin, and consequently the intracellular biochemical cascade leading to light emission by the CANARY cell will not be initiated.

The problem of soluble toxin detection can be over-

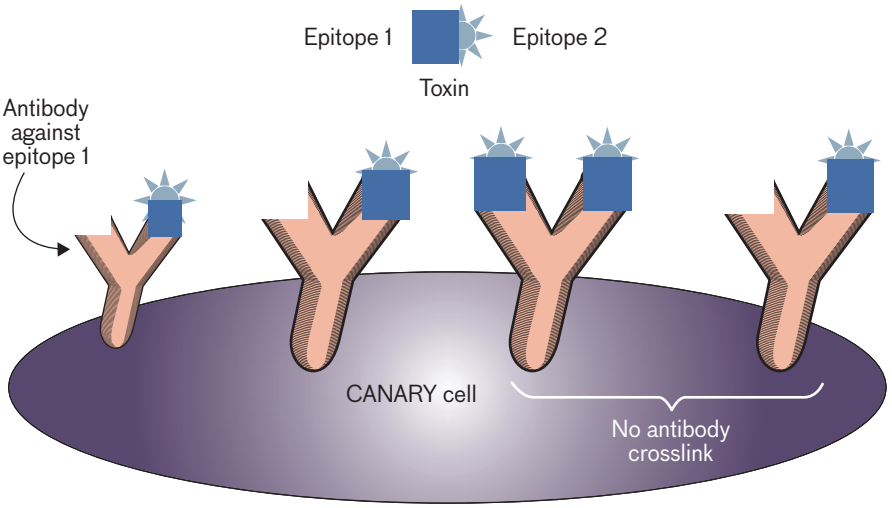


**FIGURE 9.** Protein toxins are large enough to simultaneously bind to different antibodies, but each individual antibody has only one binding site (epitope) on each toxin molecule. In the example shown, the toxin contains two epitopes. However, the CANARY cell expresses only one antibody. While the antibodies on the surface of the CANARY cell can bind to the toxin, the antibodies are not crosslinked by the toxin and can still diffuse laterally in the cell membrane. This crosslinking is required to initiate the intracellular response that leads to light emission.

come by immobilizing many copies of a toxin before they are presented to CANARY cells. There are several techniques that can be used to nonspecifically immobilize most or all of the proteins in a solution, including acid treatment to precipitate the proteins, or strong ion-exchange resins on which to adsorb most of the proteins. However, many complex test matrices, such as serum or milk, are very protein rich. Serum proteins (typically at a concentration of about 80 mg/mL) could be in such high excess over lethal amounts of toxin (botulinum toxin is lethal to humans in midng levels) that detection of toxin in this overwhelming background would be very difficult. The answer is to selectively immobilize the target toxin and wash away contaminating proteins.

One very effective method to specifically immobilize a toxin is to capture it on beads coated with antibodies against that specific toxin. The antibody-coated beads are incubated in a solution suspected of containing toxin, and washed to remove contaminating proteins and other material. The toxin-decorated beads can then present purified, immobilized toxin to CANARY cells, as illustrated in Figure 10. These CANARY cells must express an antibody that binds to the toxin at a different site from that of the capture antibody. Because the toxin is immobilized on the bead, the antibodies on the CANARY cell that bind to the toxin are also immobilized, and therefore light emission is stimulated.

This approach has been used to develop a very effective CANARY assay for botulinum neurotoxin type A

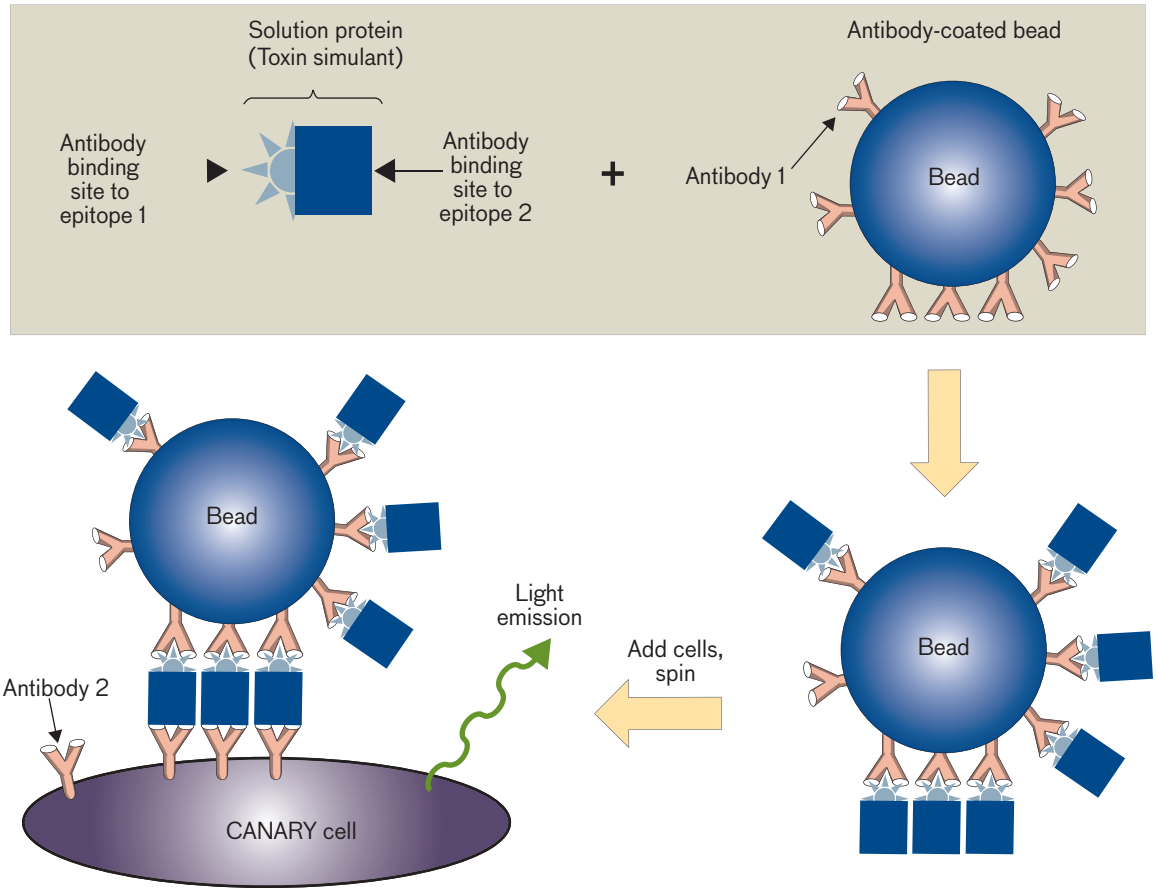


**FIGURE 10.** Two antibodies are used to assay for toxins. Antibody 1, which is attached to magnetic beads, binds to epitope 1 on the toxin molecule. Antibody 2 binds to epitope 2, and is expressed by CANARY cells. A solution containing toxin is added to the beads. Toxin attaches to antibody 1, so that the bead is decorated with toxin immobilized on its surface. Antibody 2 on the surface of the CANARY cells binds to these toxin-decorated beads. Because the toxin on the bead surface is immobilized, the antibodies on the CANARY cell are also immobilized. If the density of toxin is high enough, the CANARY-cell antibodies are crosslinked, and the CANARY cell emits light.

(BoNT/A). BoNT/A is the most poisonous toxin known to man, with an $LD_{50}$ (lethal dose for 50% of the people) for a 55 kg adult of about 550 ng by inhalation. Under ideal conditions, the assay sensitivity is currently 16 pg (1.6 ng/mL). The entire assay, including a two-minute binding step, magnetic capture, bead washing, cell addition, and light output measurement, takes about six minutes. Significantly, the use of capture beads facilitates the transfer of toxin from cell-incompatible solutions (containing nonspecific stimulators or inhibitors of CANARY cells) into CANARY-cell-compatible solutions. This transfer greatly expands the types of matrices in which CANARY can be used to detect toxins. However, some solutions, particularly those which contain high concentrations of extraneous proteins like serum and milk, still inhibit the assay to some extent. In these solutions, the sensitivity of CANARY to BoNT/A is decreased by fivefold to about 80 pg (8 ng/mL). Table 2 presents further details of toxicity and CANARY sensitivity to BoNT/A.

Increasing the time that the beads are allowed to capture toxin from two minutes to several hours improves the sensitivity by between 5- and 25-fold. However, even in difficult matrices the assay can still detect a small fraction of an $LD_{50}$ in six minutes, so for most applications further improvements in sensitivity would prove academic. Detection of BoNT/A is a stringent example of the sensitivity of the CANARY assay to toxins. It certainly follows that if CANARY can detect physiologically relevant amounts of botulinum toxin, it could detect physiologically relevant amounts of other, less toxic substances such as ricin.

### DNA Sequence Detection

The detection of soluble macromolecules has a second interesting application: identification of DNA and RNA sequences. The ability to identify nucleic acid (NA: DNA or RNA) sequences is important for at least two reasons. First, in an age when the entire SARS genome was sequenced within weeks of the virus' identification, it is probable that the first hard information available concerning a new or genetically modified pathogen will be its NA sequence. Production of monoclonal antibodies takes months, but production of nucleic acid probes takes hours. It is important to develop assays that have the flexibility to respond quickly to new threats. A second advantage of NA probes is that they examine the actual genetics of the target organism. Genetic differences can be critical in discriminating a harmless bacterium or virus from a closely related but lethal pathogen. Antibody-based assays often cannot make this distinction.

The assay uses a single CANARY cell line that expresses an antibody against digoxigenin. Once the NA sequence of a pathogen is determined, multiple short probes are synthesized that bind adjacent to each other along a specific sequence on the target NA. Each of these probes is labeled with a single digoxigenin molecule. If these probes are added to solution containing the target NA sequence, the binding of multiple digoxigenin-containing probes

**Table 2: Toxicity and CANARY Detection for Botulinum Neurotoxin Type A (BoNT/A)**

| BoNT/A $LD_{50}$ | ORAL 1–$\mu$g/kg | INHALATION 10–13 ng/kg |
|---|---|---|
| $LD_{50}$ for a 55 kg person | 55 | 550 |
| Portion of $LD_{50}$ detectable by CANARY | $1.45 \times 10^{-6}$ | $1.45 \times 10^{-4}$ |
| $LD_{50}$ per 80 pg toxin (Approximate limit of detection [LOD] in serum) | $6.87 \times 10^{5}$ | $6.87 \times 10^{3}$ |
| Approximate LOD | 1 $LD_{50}$ per 6.9 liters of milk | 7 agent-containing particles* |

*An agent-containing particle was presumed to be a dry sphere 3 microns in diameter and composed of 10% BoNT/A. At this formulation, 49,000 particles would contain one $LD_{50}$ for a 55 kg person. $LD_{50}$ is the lethal dose for 50% of the people.

produces a tight cluster of immobilized digoxigenin molecules, shown in Figure 11, which will stimulate light production from the CANARY cell. In the absence of target NA, each digoxigenin-labeled probe remains monomeric, and therefore cannot crosslink antibodies on the surface of CANARY cells. We used these procedures to detect RNA from a gene responsible for bacterial resistance to ampicillin with probes specific for that RNA. Nontarget RNA does not bind to the probes, and therefore does not stimulate the cells.

There are advantages to detecting RNA as compared to DNA. First, while there is only one copy of genomic DNA per bacterium, there can be thousands of copies of a single RNA, so the number of target molecules per bacterium is much higher. Second, because probe binding requires that the target NA be single stranded, a denaturing step must be performed to separate the two constituent strands of DNA. RNA, however, is normally single stranded. Third, because RNA degrades rapidly, it is often used as a viability test for bacteria. Viability tests can be very important in determining whether a putative attack contains live agent, or whether a decontaminated area has actually been rendered safe. The limit of detection for this assay is about 20 ng. While this level of sensitivity is not sufficient for a typical aerosol sensor, it would be sufficient for identification and characterization of organisms in materials in which the amount of agent is large, such as weapons contents, fermenter residues, and white powders. While not nearly as sensitive as PCR, DNA detection by CANARY would be faster and less expensive, and could be more highly multiplexed in a small fieldable instrument.

## Aerosol-Collection Techniques

Dry-aerosol-collection technologies specifically tailored for the CANARY assay have been developed to take full advantage of the assay speed. Unlike many other air-collection systems that require wetting agents and complicated fluidics, the dry-impaction system collects particles directly from the air onto a dry surface, thereby eliminating almost all consumables from the process. In addition to the low material consumption of this impaction system, it does not suffer from the low-temperature freeze-out experienced by liquid-based collection systems.

This simple collection method separates pathogen particles from the airstream by exploiting the relatively

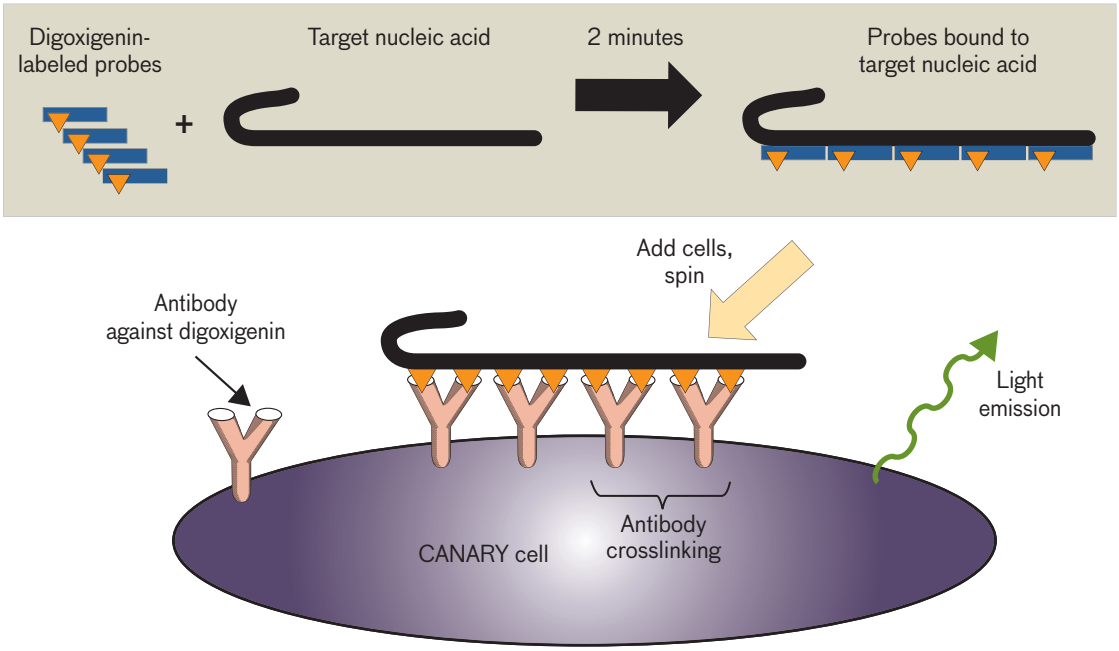


**FIGURE 11.** Several DNA probes are designed that bind to a specific region on a single-target nucleic acid (NA). Each of these probes is labeled with one digoxigenin molecule (orange triangle). In the presence of target NA, the binding of multiple probes forms a cluster of digoxigenin molecules held together by the target NA. Addition of this probe-target complex to CANARY cells that express antibody against the digoxigenin molecule results in antibody crosslinking and light emission.

high momentum of particles to force them to impact on a dry surface where a fraction of the impacted particles are retained. The basic concept and one of our collector prototypes are shown in Figure 12.

An ideal aerosol impactor shows little or no collection of very small particles (which can follow the diverted air stream), or large particles (whose momentum takes them out of the air stream earlier in the input plumbing), but good efficiency of capture for particle sizes between these extremes. Impactors are typically characterized by the particle size at which 50% collection efficiency occurs. Figure 13 shows that for this prototype tube impactor, 50% collection efficiency ($D_{50}$) occurs at approximately 1 $\mu$m diameter at a flow rate of 5 liters per minute (this flow rate is readily achievable for applications requiring low power consumption). Collection of larger numbers of particles can be accomplished easily by increasing the sampling rate or time.

Since dry impaction localizes bio-agents to the tube surface, it eliminates the need to pre-spin the sample for maximum performance. This localization allows the CANARY assay protocol for dry sample identification to be much faster and simpler to perform (and automate) than the protocol used for liquid samples (Figure 14). Identification of dry samples also has the potential to provide improved overall sensitivity to small viruses and other pathogens that are not readily sedimentable in the liquid assay, because all collected particles will be adhered to the bottom of the tube during impaction regardless of the size of the individual pathogens incorporated in the aerosol particle.

To demonstrate the efficacy of the dry-impaction collection technique for the CANARY sensor application, we aerosolized individual *Bacillus subtilis* (*Bs*) spores with a Collison nebulizer and collected them in the prototype shown in Figure 12 for 30 seconds at 5 liters per minute. The B cells were added directly to the sample-containing tube, placed in the portable CANARY apparatus, and spun for 5 seconds. Then the light signal was quantified with a PMT. The results are displayed in Figure 14 and show that the direct-impaction technique yields a B-cell response that is similar in kinetics to the pre-spun liquid samples.

This proof-of-concept experiment, with an overall response time of 1 minute (30 second collection followed by peak photon intensity in less than 30 seconds of analysis time), demonstrates that CANARY has the potential to improve the combined speed and sensitivity for bio-aerosol identification by more than an order of magnitude compared to all other automated bio-aerosol identification sensors. CANARY sensors provided the first (and still the only) demonstration of the potential for detect-to-protect biodefense capability in a biological identification sensor (Figure 1). This unique demonstration motivated the rapid development of automated bio-aerosol sensors, enabling the technology to leave the laboratory and operate in real-life environments.

### Automated CANARY Bio-Aerosol Sensor

To demonstrate detect-to-protect capability, we seamlessly integrated the CANARY identification technology with the dry-aerosol-collection architecture in two first-generation sensors, the Biological Agent Warning Sensor CANARY (BCAN) and the Triggered CANARY (TCAN).

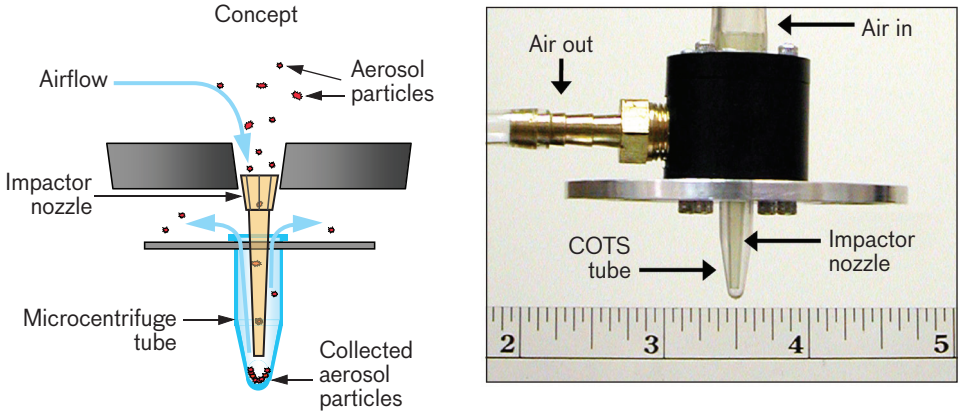


**FIGURE 12.** The dry-impaction concept (left) is implemented by the prototype device for collecting aerosol particles shown on the right. Several prototype tube impactors have been created by using COTS microcentrifuge tubes and various impactor nozzles, including pipette tips (as in this figure) and hypodermic dispensing tips. Inexpensive single-use impactors are envisioned that would include a downstream filter to prevent contamination of the (reusable) air pump that maintains the airflow through the impactor. This design is readily scalable for parallel collection into multiple tubes. After use in the CANARY assay the individual tubes can be sealed and retained for use as confirmatory samples.

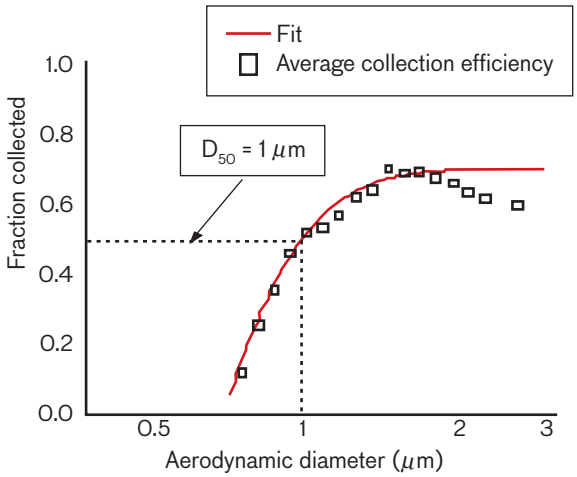


**FIGURE 13.** The data points represent the average efficiency measured in the device shown in Figure 12 from six impaction runs with polydisperse polystyrene-latex spheres aerosolized with a Pitt generator. The red line is an exponential fit to the averaged data between 0.7 and 1.5 μm. Above 2 μm, the calculated efficiency numbers become less reliable, due to the lower aerosolization efficiency (and therefore low particle counts) for these larger particles in the Pitt generator.

The BCAN sensor was designed to provide 30 automated sampling and analysis cycles, with sensitivity sufficient to detect low-concentration threats. We tested the sensor extensively to establish receiver operating characteristics (ROC) curves characterizing CANARY performance and false-positive rates in a variety of real-life environments. The excellent performance characteristics demonstrated by the BCAN sensor motivated the development of TCAN, a simplified CANARY sensor tailored to meet the less demanding requirements anticipated for indoor bio-aerosol monitoring.

## BCAN Sensor Development and Testing

The first step toward developing any automated CANARY bio-aerosol sensor was to design a reliable way to combine the dry collection with a centrifuge-enhanced CANARY assay. Furthermore, since fluidics systems were not needed for aerosol collection, we constrained our design of cell delivery systems to those which didn't require fluidics mechanisms. The main benefit to this approach is the complete elimination of a system that accounts for much of the high cost, increased size and complexity, and reduced reliability of other bio-aerosol sensor platforms. Instead, the BCAN sensor utilizes simple carriers incorporating appropriate aerosol collection features and individual aliquots of B cells stored in COTS capsules that, after collection, release their contents automatically during a brief spin. The key details of this design are outlined in Figure 15.

Each BCAN carrier contains four parallel mechanisms (or channels) that provide the four core functions necessary for CANARY analysis: cell storage, aerosol sampling, cell delivery, and signal transmission to PMTs. The BCAN testbed, shown on the left side of Figure 16, can house and automatically process up to 25 of these carriers between reloading. By using *Bs* spores as a simulant for anthrax we demonstrated that this first-generation automated sensor could provide greater than 96% probability of identification for bio-aerosols at concentrations of greater than 100 agent-containing particles per liter of

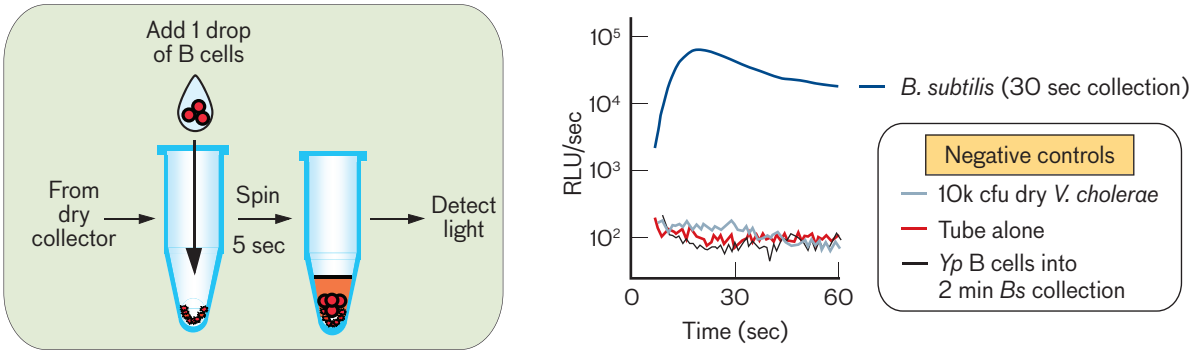


**FIGURE 14.** Dry identification of *Bacillus subtilis* (*Bs*) spores is performed, as shown in the schematic of the dry-assay protocol (left). B cells specific for the anthrax simulant *Bs* are added and a brief centrifugal spin drives the cells to the collection site at the bottom of the sample tube. Specificity demonstrations of the dry-assay format (right) show a clear response of the CANARY cells in detecting *Bs* impacted onto sample tubes, while negative controls—other pathogens, empty tubes, and alternate B cells—are consistently negative.

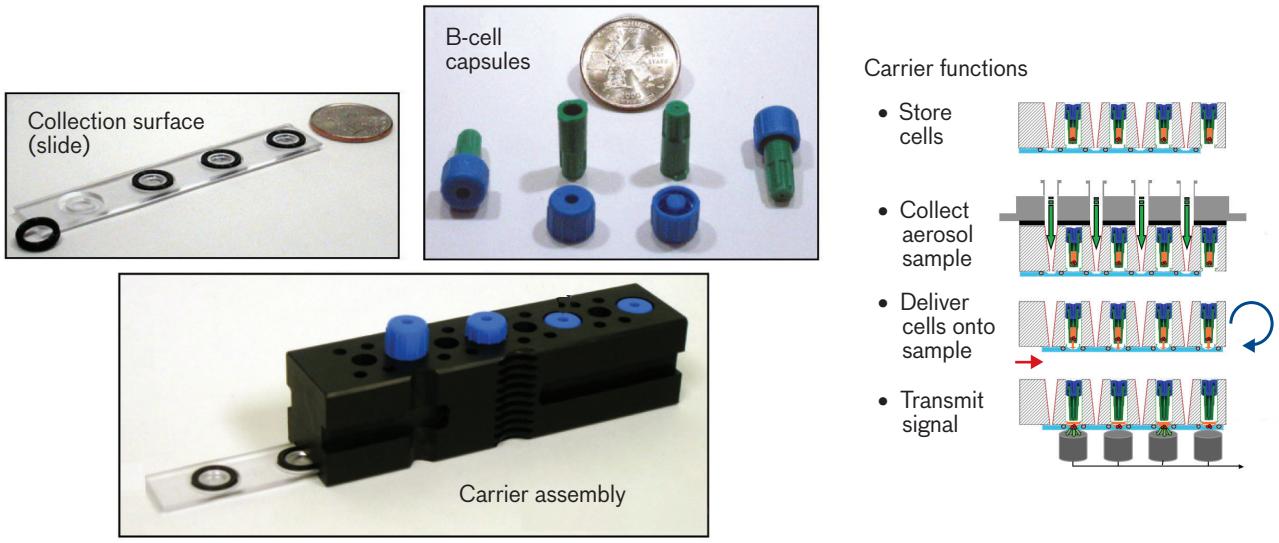


**FIGURE 15.** The Biological Agent Warning Sensor CANARY (BCAN) carrier performs all the functions of automated bio-aerosol collection and CANARY analysis. B cells are stored at 20°C prior to use. Aerosol samples are collected through the carrier interface plate, which is sealed to the top of the assembly. Flow equalization is maintained in this step. After aerosol collection, the glass plate is translated, placing the samples under the B cells, and the cells are delivered onto the sample. The unit is spun at 7500 rpm to mix and concentrate the cells and the sample. Photon counters are placed below the transparent glass slide to detect the signals from the CANARY cells.

air (ACPLA) with a 3 min response time. Furthermore, the ability to operate this sensor in a variety of indoor and outdoor locations enabled the potential interference from background aerosols in realistic environments to be defined. Over 13,000 tests were completed in nine different locations spanning a wide range of background conditions to establish the number of anomalous positive signals (false positives) that could be expected from this sensor technology. Results from this field data were combined with the *Bacillus subtilis* data to generate ROC curves illustrating the relationship between probability of detection and the probability of false positives in various environments. Typical results for cases in which only one test for a particular threat is required to go positive to

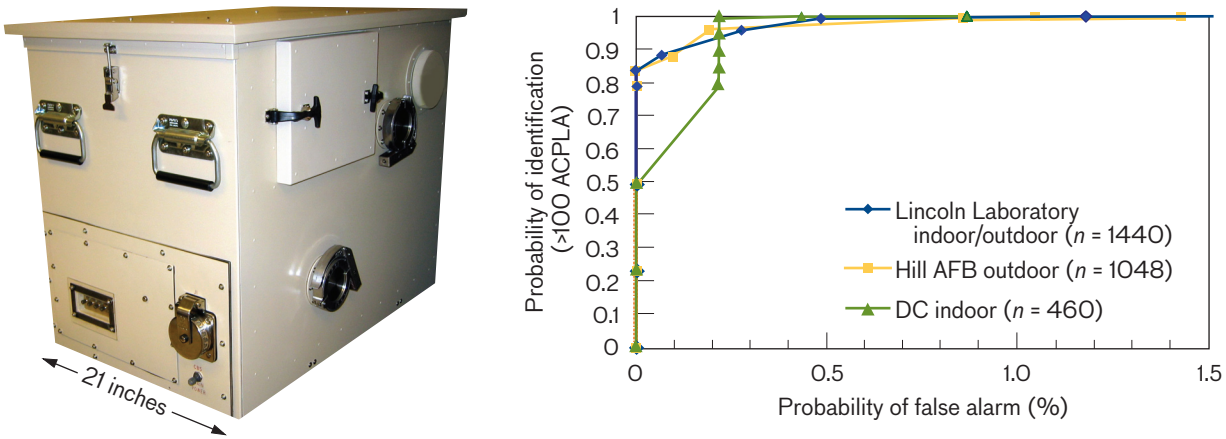


**FIGURE 16.** The transportable BCAN sensor, shown on left, produced the data for several cases typical of CANARY analysis of a particular threat (an indoor/outdoor study of Lincoln Laboratory, an outdoor test at Hill Air Force Base, and an indoor test in a District of Columbia office building). The resulting receiver operating characteristics (ROC) graph on the right shows the comparison between false-alarm rates and the probability of identification on the basis of a single test. In each curve, *n* is the number of tests at that specific test site. ACPLA stands for agent-containing particles per liter of air.

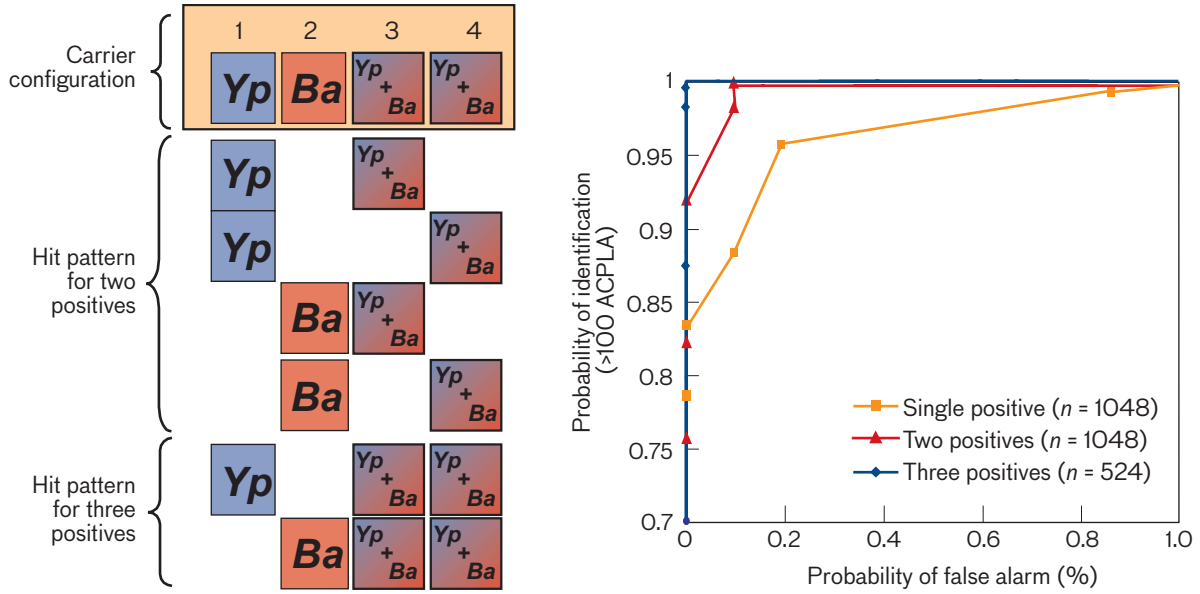


**FIGURE 17.** Introducing test redundancy enables further reductions in the probability of false alarm while maintaining excellent probabilities of identification. For a 4-position carrier, channel 1 analyzes for *Yp*, channel 2 for *Ba*, and channels 3 and 4 for both *Yp* and *Ba*. In this method, the presence of *Yp* or *Ba* might trigger either two or three positives. The previous single-positive test is surpassed in analysis by both two- and three-positive tests results.

declare an alarm are shown in Figure 16. Only the D.C. indoor test shows an increase in false-positive rate for probabilities of identification less than 90%.

Assay multiplexing can be achieved by placing either multiple B-cell lines or individual B-cell lines expressing multiple antibodies into a single channel. Such a system minimizes hardware complexity (and size) and can theoretically identify $2n - 1$ agents (where $n$ is the number of channels) in a single-agent attack scenario. The limitation of multiplexing with more than one cell line per channel is that sensitivity is diminished when using more than 3. In addition to expanding the number of identifiable agents, multiplexing can provide redundancy, thereby reducing the false-positive rate, as shown in Figure 17 for the Hill Air Force base data of Figure 16.

An extensive set of measurements and fieldings demonstrated BCAN's capability to identify bio-aerosols at biologically relevant concentrations in as little as 90 seconds. This response time is an order of magnitude faster than any other integrated bio-aerosol identification sensor and is the only demonstration of speed consistent with the needs of detect-to-protect operation for biological defense. Perhaps even more important is the low false-positive rate established in real-life environments, 0.2% – 0.3% for single tests, and 0.1% or less for two-fold or greater redundancy, while maintaining greater than 96% probability of identification, demonstrating that this technology is ideal for systems demanding low false-alarm rates and superior speed for bio-aerosol identification. While the BCAN was designed to be a demonstration testbed, other sensor architectures offer potential advantages for customized applications. For example, TCAN sensor development was begun as a parallel-sensor development effort to establish CANARY performance for building protection.

### TCAN Sensor Development and Testing

The TCAN is a CANARY-based biosensor developed to be a simple, cost-effective means for real-time monitoring of bio-aerosols in an indoor building environment (Figure 18). This particular sensor was designed to combine both aerosol collection and B-cell delivery into an integrated radial disc format (Figure 19). The disc is designed to interface with a manifold that separates particulate-laden airflow into four separate channels. Inertial impaction techniques are then used to localize these particles into the bottom of clear disposable tubes (Figure 19). After collection of aerosol particles, valves located within the disc are opened, and the disc is centrifuged at 2000 rpm for 5 seconds. This spin step uses centrifugal force to quickly

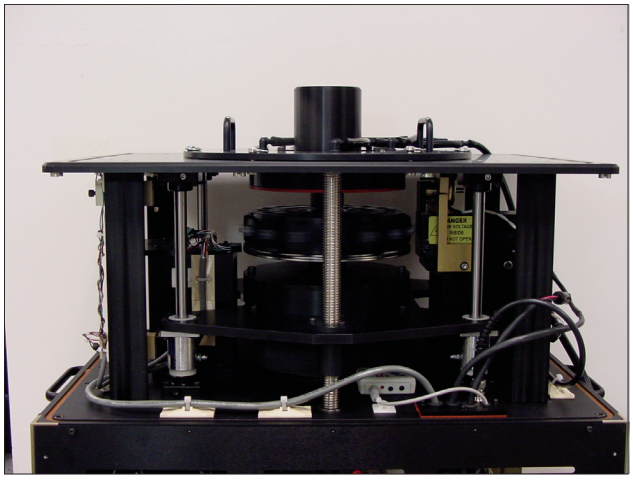

**FIGURE 18.** The Triggered CANARY (TCAN-2) automated biosensor, shown with its light-tight cover removed, contains a radial disk that handles all the CANARY analytical steps.

drive the B-cell liquid into contact with the collected particles. A single PMT is then used to identify potential bio-agents as the disc rotates. This process of aerosol collection and B-cell delivery can be repeated several times, allowing multiple CANARY assays to be performed in a single disc.

The TCAN sensor is currently part of a tiered-sensing network comprising an array of independent trigger sensors and an aerosol manifold that transports suspect particulates to a central identification location. The tiered-sensing network utilizes the speed and sensitivity of the CANARY assay to deliver high-confidence identification of suspect particles in less than 5 minutes. Results of the TCAN sensor identification are subsequently used to initiate several building heating, ventilation, and air-conditioning response measures, designed to minimize exposure and contamination of indoor building spaces.

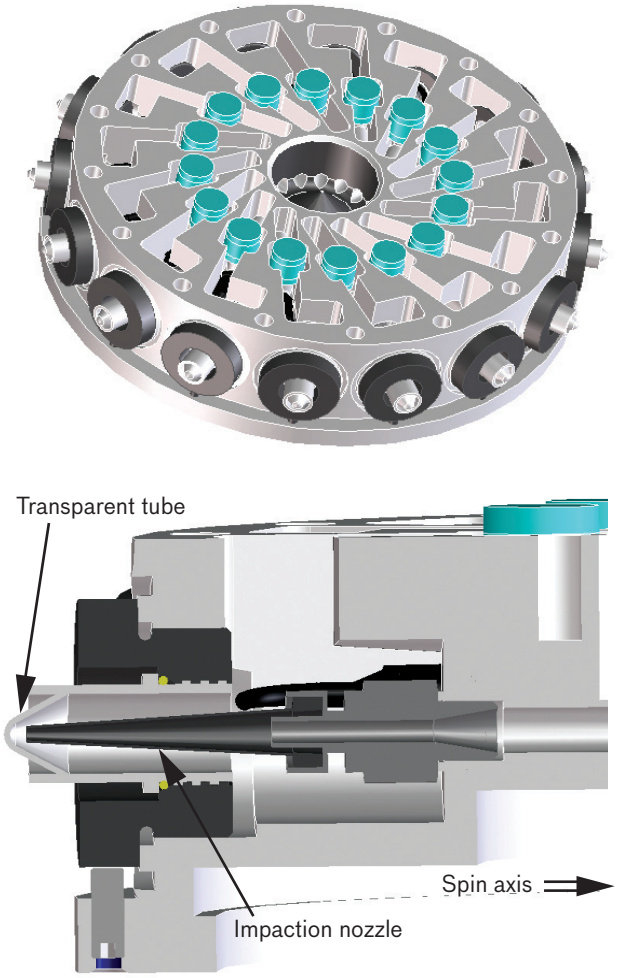


**FIGURE 19.** The CANARY disc in the TCAN-2 unit shown in Figure 18 is designed to integrate aerosol collection, B-cell delivery, and photodetection of cell radiation. The lower image is a cutaway of an individual aerosol collection module, with its impaction nozzle and transparent tube. After the sample is spun to deliver the B cells, light is detected from the B cells directly through the bottom of the tube.

## PANTHER Sensor Development and Testing

We have incorporated CANARY technology into a flexible bio-aerosol sensor platform called PANTHER (Pathogen Analyzer for Threatening Environmental Releases). The primary goal for this second generation of CANARY-based bio-aerosol sensors was to continue to improve speed and sensitivity, simplify sensor hardware, and minimize operational complexity. This goal was accomplished by incorporating the key functions of aerosol collection and CANARY analysis into simple-to-handle, inexpensive plastic disks (Figure 20) that form a common core for all members of the PANTHER sensor family. Each disk has 16 channels that are pre-loaded with a droplet of B cells to enable 16 simultaneous tests for the presence of up to 48 agents (assuming three cell types per channel) to be performed on a single aerosol collection.

The ultimate PANTHER sensors that use these disks are intended to be used individually or in networks to provide site/building protection, emergency response, rapid screening, and environmental monitoring. We have demonstrated high-confidence identification in less than two minutes of very low-concentration bio-aerosols by using


**FIGURE 20.** The Pathogen Analyzer for Threatening Environmental Releases (PANTHER) disk (left) is a self-contained bio-aerosol sampling and CANARY analyzing tool that can be loaded like a CD into the portable PANTHER CUB sensor (center) or ultimately into an autonomous PANTHER point-detection and identification sensor (right). The Compact Unit Biosensor (CUB) sensor is designed to hold and process one disk at a time, whereas the full PANTHER sensor will hold and process up to 30 disks.

the first PANTHER sensor, a portable unit referred to as the CUB (for Compact Unit Biosensor) that is 37 lb and approximately 1 $ft^3$, and can ultimately be made for less than $20,000. The design is simple and reliable: it has no fluidics, has minimal moving parts, loads like a CD player, and automatically collects and analyzes the sample.

The CUB sensor was an outgrowth of a project that initially focused on the development of an autonomous CANARY-based sensor that could exceed present and future needs for U.S. military bio-aerosol point-detection sensors. It was intended to demonstrate the opposite end of the sensor complexity and capability spectrum: a small, inexpensive, portable sensor that could automatically process a single PANTHER disk. The CUB sensor was designed, fabricated, and tested in six months. Compared to the first-generation automated CANARY bio-aerosol sensors, the CUB offers improved speed and sensitivity in a much smaller and less expensive sensor while maintaining a very low false-positive rate in real-life environments (Figure 21).

To establish sensor sensitivity, we collected aerosols of *Bs* spores produced by a Collison nebulizer for one-minute and analyzed them with cells specific for the spores in the CUB. Approximate ACPLA levels produced by each dilution were measured with a particle counter and are shown in the legend of Figure 21. The 1:8000 dilution generated a number of particles per liter that were indistinguishable from the chamber background produced when deionized water was added to the nebulizer, but should theoretically contain five spores per liter of air. Even at this extremely low concentration, a one-minute collection produces a detectable signal in a majority of the tests.

The sensitivity data from the chamber studies were then combined with background measurements made in the Lincoln Laboratory atrium over a one-week period (>1000 tests) by using cell lines specific for *Yp* and *Ba*. A computerized detection algorithm was applied to the combined data set and the probabilities of detection ($P_D$) and the correlated probability of false alarm ($P_{FA}$) were established over a range of detection threshold settings. The resulting ROC curve is shown in Figure 21 and demonstrates better than 98% probability of detection for concentrations greater than 20 ACPLA with a corresponding false-alarm rate of less than 0.1%. This preliminary performance compares quite favorably with the first-generation BCAN sensor performance and still has the potential to be optimized further with additional hardware refinements and algorithm development.

During four years of bio-aerosol-identification sensor development sponsored by three different programs, we have built and tested two generations of bio-aerosol sensors. These sensors have consistently demonstrated CANARY to be the only technology capable of providing detect-to-protect capability for bio-aerosol identification. Key features of the two first-generation sensors targeting point-detection and building defense applications

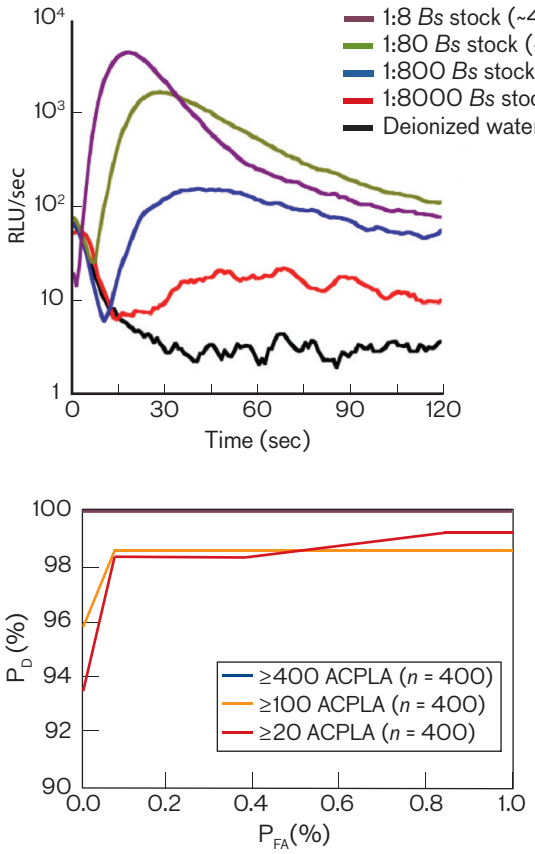


**FIGURE 21.** Preliminary performance characteristics of the PANTHER CUB sensor include (top) typical signals from CUB analysis of *B. subtilis* spore aerosols; (bottom) a ROC curve for the CUB sensor operating in a typical high-traffic indoor environment. We tested the chamber against various concentrations of Collison-generated spores and deionized water as a negative sample. The bottom data show that throughout over 1700 tests, the single-test probability of false alarm was less than 0.1% with a greater than 98% probability of detection for all ACPLAs tested.

have been combined into a single technology platform, PANTHER, that offers order-of-magnitude improvements in speed, sensitivity, weight, and cost compared to fielded bio-aerosol identifiers. Designs incorporating the core PANTHER analysis module into stand-alone, autonomous point sensors and small, portable bio-aerosol sensors (PANTHER CUB) have been completed, and CUB prototypes have demonstrated reliable identification of anthrax spore simulants at concentrations below 10 ACPLA with a two-minute response time. The ability to provide this level of performance in a small package costing less than $20,000 demonstrates the potential for CANARY technology to transform biodefense capabilities in the near term.

## Future Work—Cell Storage

One important obstacle to overcome in order to achieve broad user acceptance is that of long-term storage of the B-cell reagent. The cells are now kept frozen or refrigerated until ready to use, which is acceptable (though not optimal) for many medical and homeland security environments, but is unacceptable for forward-deployed military units. In addition, most users desire a B-cell reagent that can sit on a shelf in a warehouse or laboratory at ambient temperature for six months up to several years and that can be taken out, loaded into a sensor, and used for a test.

Until recently, the shelf life of the standard CANARY cell reagent was two days at room temperature, two weeks at 4°C, and several years frozen. Experiments comparing stored cells to freshly prepared cells indicated that the loss of activity during storage was due not only to a decrease in cell viability, but also to a decrease in the amount of light emitted per cell in response to pathogens. We have therefore begun a program to improve both the viability and the activity of cells stored long term at room temperature and 4°C by using additives such as antioxidants as well as by inserting preservation genes into the B cells, including those from extremophiles (organisms that can tolerate extremes of temperature or desiccation). The most promising results found to date for cell storage at 4°C were obtained by overexpressing a cell-preservation gene known as Bcl-XL (Figure 22). This work is still in its early stages, but we have so far extended the shelf life of CANARY cells to one week at room temperature and 1.5 months at 4°C. We have shown that while protective additives and treatments provide some benefit, genetic manipulation has been the more successful approach. Future work will combine genes that are believed to work in a synergistic manner to further improve storage and logistics.

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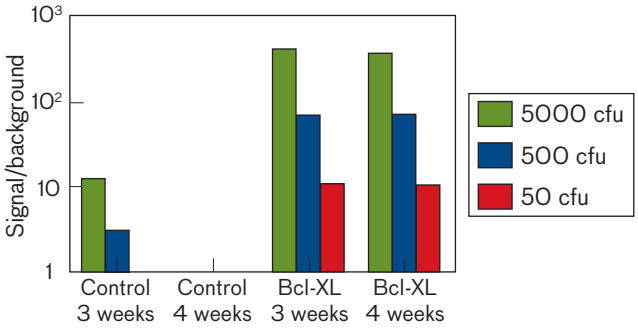


**FIGURE 22.** The cell-preservation gene Bcl-XL significantly improves the active life of the CANARY B cells. Even at the lower concentration levels (shown in red), the detected signals are nearly identical after three and four weeks.

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