Chapter 10
Microfluidic Applications in Biodefense

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Abstract

There is an increasing need for compact biodefense devices that work autonomously and consume minimal reagents. These requirements can be well met by microfluidic technologies. This review first describes the needs for biodefense, which include protection of civilian populations with detect-to-warn and detect-to-treat modalities, and the needs of the military. The different microfluidic technologies applied to each step of threat detection are then discussed. The technology areas covered are microscale

sample preparation, immunomagnetic separations, immunoassays, toxin detection, proteomics, microarrays, and microelectrophoresis. For each technology area, the potential microfluidic solutions are introduced and current relevant examples are described. For each area, the potential applications to biodefense are detailed. The present state of fully integrated microfluidic devices is reviewed. Finally, perspectives for the future are discussed.

10.1 Introduction

The growing threat of bioterrorism attacks, combined with repeated outbreaks of emerging infectious diseases, underlines the importance of infrastructure improvement for the detection and diagnosis of biowarfare agents and emerging pathogens. The growing threats posed by terrorists and rogue nations—as evidenced by Iraq's acknowledgement following the first Gulf war that it had loaded biological weapons, and multiple biological "incidents" worldwide—has raised serious concerns about bioterrorism attacks directed against the United States and other nations.

Following the October 2001 anthrax attacks in the United States, biodefense has become an area of utmost national and international urgency [1-2]. This incident sparked the testing of tens of thousands of samples for the presence of anthrax, straining the Laboratory Response Network (LRN) system. The New York City experience after the anthrax attacks is telling [3]. The increase in incoming samples went from one every several months to about 6,000 in two weeks, requiring a coordinate growth of analytical staff and laboratories by over twenty-fold. The operational expectation had been that any surge would primarily be composed of human clinical samples; instead most of the samples were environmental. A recent study of acute care facilities in Mississippi found that the diagnostic capacity of hospitals would be overwhelmed by a weapon of mass destruction (WMD) attack [4]; similar conclusions regarding the lack of diagnostic surge capacity in alternate locales were reached in other studies [5-6].

The destructive potential of genetically engineered bioagents is huge. Toxic genes can be hidden in innocuous organisms and expressed at high levels. Expression timing and genotypic specificity could be controlled to maximize impact and potentially limit spread to a defined racial pool. The purported accomplishments of the Soviet bioweapon program [7] accentu-

ate how real the problem is: sophisticated state-sponsored bioweapons programs have already genetically engineered bacteria and viruses to increase their devastating impact on human populations. “Capitalizing” on post-1972 advances in biotechnology such as genetic engineering, the Soviet Union program researched and produced a range of weapons employing smallpox, anthrax, plague, and other dangerous pathogens [8]. Fortunately, terrorists and rogue states have not yet fully incorporated biological weapons into their arsenals, to our knowledge [9]. The detection of bioengineered organisms presents even greater challenges than detecting conventional pathogens, and may require multi-tiered screening, including high resolution detection of target genes and DNA sequencing.

Similar to the needs for biodefense of engineered organisms, naturally emerging infectious diseases present another major threat to human health, through the natural spread of these organisms, their rapid evolution to human hosts, and the potential for bioterrorism using these agents. The recent outbreaks of severe acute respiratory syndrome (SARS) and bovine spongiform encephalopathy (BSE), commonly known as mad-cow disease, have raised global concerns on the need for rapid identification of causative agents and infected individuals before the virus spreads beyond control. Hantavirus pulmonary syndrome and West Nile virus are examples of additional infectious diseases now emerging in the US. Rapid molecular diagnostic methods and monitoring platforms that can adaptively be configured for newly emerging infectious diseases or newly engineered bioagents will be essential for combating these diseases and for biodefense applications.

Past incidents and the dangers of future bioterrorism attacks highlight the critical need for improved field- and laboratory-based systems to detect, identify, and subtype bioagents [10-11]. As will be seen, the state-of-art of biodefense systems today is operational but rudimentary: all US mail is screened at sorting centers. There is a strong demand from the U.S. government for next-generation systems for civilian and military applications. Biodefense monitoring equipment has even more stringent requirements: the size of the current equipment, and the acquisition and operating costs place severe constraints on widespread implementation and deployment. New analytical systems are needed that are scalable, more automated, and capable of rapid deployment in response to surging needs or field operations. The equipment needs to be smaller, use less reagents, be simpler to use, more integrated, and automated—all attibutes of microfluidic systems, making microfluidics an ideal platform to fulfill biodefense needs.

This review summarizes approaches to detecting and characterizing biological threat agents for both civilian and military biodefense; describes biodefense programs in place and under development; and delineates some of the approaches where microfluidics is presently being applied to monitor, detect, and characterize biothreat agents. Some of the primary needs are outlined, and the challenges to design and build fully integrated microfluidic systems are described. The majority of the review surveys microfluidic technologies that might or could be used in future biodefense systems. Sections cover microscale sample preparation methods; immunomagnetic separations and immunoassays; proteomics; polymerase chain reaction (PCR), quantitative PCR (qPCR) and other nucleic acid amplification methods; DNA microarrays, microelectrophoresis, and finally integrated Lab-on-a-Chip systems.

10.2 Biodefense Monitoring

There are two basic biodefense detection approaches: detect-to-warn and detect-to-treat. Detect-to-warn systems aim to identify biothreats rapidly enough to provide sufficient warning to prevent exposure by the threat. Detect-to-treat systems aim to identify the causative agent for diagnostic purposes and thereby to direct healthcare workers to the most effective treatment as quickly as possible. For all systems, low false alarm rates (FAR) and affordable acquisition and operating costs are essential for widespread adoption.

10.2.1 Civilian biodefense

Civilian biodefense is based upon surveillance to detect biothreat agents, response networks to warn and direct the treatment of the affected population [12], and the development of countermeasures [13]. Bioterrorism incidents, releases of a bioagent in a form that can harm individuals or larger populations, can range from mailing of toxin or bacteria, to release of aerosols in high profile events, to attacks on the food supply. BioShield, a countermeasures program [14], is a 10-year, $5.6 billion U.S. program for the advanced development and purchase of medical countermeasures. Acquisition programs have been announced to counter *Bacillus anthracis* (anthrax), Variola virus (smallpox), botulinum toxins, and radiological/nuclear agents.

The primary programs that have been implemented for civilian biodefense detection include screening of all postal mail at sorting centers, the BioWatch program, selected localized screening in subways and other undisclosed locations, and the LRN. The currently deployed systems principally use full volume or meso-scale fluidics. Despite the need for more advanced detect-to-warn biodefense detection for the general public, these systems have largely remained undeveloped in large part due to the complexity of integrating the complete process.

The largest monitoring program is BioWatch [15-16], a joint effort by the Department of Homeland Security (DHS), the Centers for Disease Control and Prevention (CDC), and the Environmental Protection Agency (EPA). BioWatch is a 'detect-to-treat' program and monitors the air in at least 31 cities (and as many as 120 cities) [17] for significant release of bioagents. DHS does not report cities monitored by the BioWatch program or the assays used.

In 2004 and 2005, DHS funding for the Biowatch was $26.8 million for the EPA and $28.5 million for the CDC [18]. The EPA is responsible for continual air sampling by aerosol collectors that trap airborne particles onto filters. The filters are collected for analysis every 24 hours. The CDC is responsible for the analysis of the filters at state and local public health laboratories, and developing new protocols in coordination with both the Department of Energy (DOE) national laboratories and the EPA. The assays are generally acknowledged to be PCR amplified detection of specific targets using classified primer sequences. The list of target agents is similarly classified but thought to include at least anthrax, smallpox, plague, and tularemia. The BioWatch program has now processed over 2 million samples without a false positive [19]—an impressive accomplishment. However, the daily sampling frequency and the amount of coverage of the Biowatch program still leaves the civilian populace vulnerable.

DHS has been funding the Biological Autonomous Networked Detectors (BAND) program as a next-generation system to alleviate some of the deficiencies of the BioWatch program. The original goals of the BAND program were continuous air monitoring with sample analysis every three hours. The detection limits were 100 organisms or 10 ng of toxin per 17,000 liters of air processed, with a very low FAR. The instrument was planned to have an acquisition cost of $25,000, an annual operating cost of $15,000, and run autonomously for 30 days. Additional requirements were for dimensions of 2 ft^3 and the ability to detect up to 20 organisms and tox-

ins. The BAND participants have almost uniformly taken a microfluidics approach. Their efforts are described in more technical detail in a later section of this chapter.

In addition to detecting bioagents in the primary environment, a major effort has gone into upgrading the response of the medical community to detect unannounced attacks [20], since the expectation is that in many scenarios the first alert will be in the form of patients presenting at doctors' offices or hospitals [21]. Biodefense systems are also required to monitor food and water sources [22-23], suspect powders, the exposure of first responders, and test for decontamination after treatment of personnel, equipment, and key environments.

10.2.2 Military biodefense

The U.S. military biodefense programs aim to detect and identify biological warfare agents that an enemy might use to degrade forces, contaminate bases, and spread confusion throughout command and control systems. Various defense programs are delivering technologies that are beginning to counter these vulnerabilities. For example, the Portal Shield program is designed for facilities protection; the Joint Biological Agent Identifiation and Diagnostic System (JBAIDS) is designed for both detection and diagnostics of environmental and clinical samples [24], and the Joint Biological Point Detection System (JBPDS) is designed for detect-to-warn capabilities in the field.

Portal Shield is an array-able sensor system developed to provide early warning of biological attacks for high-value, fixed-site assets, such as air bases and port facilities. Portal Shield is designed to detect and identify threats simultaneously within 25 min. It is programable to survey continuously as well as perform random or directed sampling. Portal Shield was deployed in the Persian Gulf region in February 1998 during Operation Desert Thunder, and the current instrumentation is about two thirds the size of a typical office desk. It's fully modularized, self-contained, and can detect eight different agents. As many as 18 sensor units may be arrayed in a given area and are able to communicate with each other, so there is no reliance on just one of them sounding an alarm. Using an array system, the false positive rate diminishes towards zero.

Table 10.1 JBAIDS targeted agents [24]

Agent or Disease	Organism
Block I	
Anthrax	*Bacillus anthracis*
Brucellosis	*Brucellae*
Ebola-Marburg	*Filoviridae*
Plague	*Yersinia pestis*
Q fever	*Coxiella burnetii*
Salmonellosis	*Salmonellae*
Smallpox	*Orthropox viruses*
Tularemia	*Franciscella tularensis*
Typhus fever	*Rickettsiaei*
Block II	
Botulinum	*Clostridium botulinum*
Ricin	*Ricinus communis*
Staphylococcus enterotoxins (e.g., staphylococcal enterotoxin B)	*Staphylococcus aureus*

JBAIDS is the DoD's first common platform for identification and diagnostic confirmation of biological agent exposure or infection. JBAIDS Block I is currently operational as a real-time PCR instrument with FDA approved assays. The current JBAIDS Block I system (and JBTDS systems) utilizes manual sample preparation (disrupting the cell or spore and extracting the nucleic acid or protein of interest) which results in: 1) complex operator procedures that may result in human error, 2) increased operational costs, and 3) support and logistical requirements that preclude remote operations. The intent of JBAIDS Block III, Next Generation Diagnostics (NGD), is to establish a new system incorporating the capabilities of Block I and Block II capabilities (Table 10.1) and adding immunoassay capabilities and the ability to identify up to 50 agents including toxins in 15 minutes using automated, miniaturized sample preparation integrated with analysis for nucleic acids and proteins, in a hand held or smaller format.

JBPDS is planned to detect, identify, and warn against the presence of up to 24 biowarfare agents at discrete points within a given field environment. JBPDS is being designed with a sampler, trigger detection, and identification technologies that allow it to rapidly and automatically detect and identify biological threat agents. JBPDS was committed to initial limited production and procurement during FY2006, with planned full production in 2007. Future JBPDS improvements will reduce size, weight,

power consumption, and reagent use while increasing the number of agents recognized, sensitivity, and system reliability.

A number of the requirements of the above programs may be met through the implementation of microfluidics. For example, reduced consummable use, weight and size are directly tied to the drastic reduction of reaction volume employed in microfluidic systems and the use of miniaturized microfluidic components. Increasing the number of recognized agents can also be accomodated without high order reaction multiplexing by using multi-channel microfluidic devices with single-plex reactions carried out in parallel. In theory, with microfabricated microchips adding more channels is simple a 'cut and paste' exercise once the issues of connections and integation are solved. There are considerable issues in integrating all processing and analysis steps in a 'hands-free' device. However, microfluidics *requires* a 'hands-free' implementation once samples are loaded since typically there is nothing the user can do to intervene.

10.3 Current Biodefense Detection and Identification Methods

The detection of biothreat agents today can be segmented into laboratory detection and field detection methods, and by the type of sample matrix processed (reviewed in [11,25-28]). The detection must be sensitive, specific for the test organism(s), and may require substantial up-front sample preparation before the read-out assay can be performed.

There are a number of commercial tests available today to detect biothreats using nucleic acid, immunological, and biochemical methods. The appropriate test may be determined by the level of information required (*i.e.*, phenotypic or genotypic), timeframe, and the consequences. In general, nucleic acid tests are more sensitive, but require a higher order of skilled operators and more sophisticated equipment than immunological tests, and cannot detect toxins.

Confounding the problem is the need to assess samples for the presence of many possible biothreat agents; the detection of genetically engineered organisms potentially designed to evade standard detection methods; and the vast numbers of people crossing international borders (600 million international travelers per year [27]).

Sample matrix and sample preparation methods are key variables in biodefense detection. Targets may be contained in air, food, water, bodily fluids, powders, swabs, swipes, cloth, or filters, among many other possibilities. Significant reductions in sample volume are required and sample preparation methods must remove contaminants such as metal ions, heme, humic acids, and other compounds, which inhibit PCR or other assays. To enable input of samples into microfluidic systems, the target analyte needs to be highly concentrated without concentrating inhibitors. Sample preparation issues are further detailed in section 10.5.

10.3.1 Laboratory detection

Traditional identification of pathogens is often tedious and prolonged, involving batteries of tests that often take days or even longer to confirm. Standard clinical laboratory identification of *Bacillus anthracis* serves as an example [12]. *Bacillus anthracis* testing by a LRN Level A laboratory begins with growth of the organism, a Gram stain, capsule observation and routine culturing on sheep blood agar. This is followed by observation of colony morphology, motility, sporulation, and hemolysis. The presumptive identification can take up to 96 hours with additional days for confirmation. Confirmatory testing at Level B labs (State and Federal laboratories) consists of phage lysis and immuno-fluorescence assays of a cell wall and capsule. Level C laboratories determine antimicrobial susceptibility and apply more advanced technology including PCR, qPCR, and time-resolved fluorescence measurements. Finally, at Level D, labs (CDC), in-depth molecular characterization is performed using multiple-locus VNTR (variable-number tandem repeat) analysis (MLVA), 16S rDNA ribotyping and other methods, including sequencing to provide subtyping information for identification [29]. Full characterization can include complete genomic sequencing to identify the exact strain variant for epidemiological and forensic analysis.

Similarly the Armed Forces Institute of Pathology uses DNA extraction, DNA quantification, qPCR of unique genetic targets, 16S rRNA gene sequencing, amplified fragment length polymorphism polymerase chain reaction (AFLP-PCR), and repetitive element polymerase chain reaction DNA fingerprinting to characterize strains [30]. The results are compared to extensive databases that have been assembled [31-32].

10.3.2 Field detection

For more rapid detection in the field, current detection methods use immunoassays, PCR, and other molecular typing methods to provide information on suspected biothreat samples. Direct fluorescence assay with monoclonal antibodies to cell wall and capsule components have also worked well for *B. anthracis* [33]. Real-time PCR or qPCR methods are frequently performed [29] and can yield rapid identification in the field, using the LightCycler (Roche), GeneXpert (Cepheid), JBAIDS (Idaho Technology) or other systems. DNA sequencing of 16S ribosomal sequences, plasmids, or variable regions can yield the highest resolution identification, but is relatively slow and has a low throughput compared to other methods; sequencing has not yet been adapted to field applications.

One of the most advanced field detection concepts is qPCR packaged in portable devices. Researchers at Lawrence Livermore National Laboratories (LLNL) demonstrated real-time detection of PCR products in a miniaturized silicon reactor with thin film heaters and integrated fluorescence detection [34]. This work was extended at LLNL to a 10-channel advanced nucleic acid analyzer and a portable version was devised [35]. Cepheid developed commercial versions of this device with integrated (multi-microliter) sample processing for qPCR analysis, now incorporated into the Northrop Grumman Biohazard Detection System (BDS), used by the Postal System for monitoring mail facilities. The BDS incorporates upstream air sampling with the GeneXpert (Cepheid), which prepares DNA from a fluidized aerosol sample and then performs qPCR. The system is fully integrated, automated, and reports data to a central point.

The BioWatch program grew in part from the Biological Aerosol Sentry and Information System (BASIS) project developed at the LLNL and Los Alamos National Laboratory. BASIS used filters to collect aerosolized particulate samples at large events such as the Olympics. Filters were analyzed by PCR in separate laboratory facilities using largely manual protocols [36]. LLNL also developed the Autonomous Pathogen Detection System (APDS) [37] as a stand-alone, autonomous aerosol detection device. APDS, a podium-sized system, monitors air for all three biological threat agent types (bacteria, viruses, and toxins) by continuously performing aerosol collection, sample preparation, and multiplexed biological tests. The APDS first employs fluorescent bead-based immunoassays to detect more than ten agents. If a positive signal is detected, a second tier confirmation using qPCR is enabled. APDS systems have been field tested at major transportation centers and at special events [32].

A number of companies (*e.g.*, Tetracore, Alexeter Technologies) have developed and market lateral flow immunoassays for field detection of a number of relevant biothreat agents. These are strips of porous materials to which sample is added to one end, and during migration to the other end, encounters a region containing antibodies specific to the target along with a chromophore. While most immunoassays are not particularly sensitive, they are the normally used field screening method and are also used in situations where large numbers of negative samples are anticipated.

10.4 Microfluidic Challenges for Advanced Biodefense Detection and Identification Methods

Rapid detection, identification, and subtyping analysis of pathogenic organisms and toxins are critical needs for biodefense and for the management of emerging infectious diseases. Autonomous systems that can detect and provide initial identification of bioagents are required for field monitoring and to provide any reasonable degree of protection to civilians. Laboratories require automated systems that can rapidly genotype microorganisms from human samples, environmental samples, or food and differentiate at the strain level and better direct treatment. All of these newly developed systems must detect nucleic acids and/or toxins in varying amounts, formats, and in many different matrices. They will need to be completely automated or simple to use; incorporate advanced technologies including sample preparation starting from primary samples (aerosols, blood, etc.), molecular detection, automation, microfluidics, and bioinformatics; reduce reagent consumption and space requirements; and provide cost and performance advantages compared to present systems. Analytical techniques such as PCR, VNTR, MLVA, AFLP, and single molecule detection are well suited to analysis on microfluidic systems. In addition, the systems should be capable of accommodating new assays as they become available.

The burgeoning field of microfluidics can offer remedies that fulfill many of these needs and is thus becoming an ever increasingly desired component of next-generation systems. Microfluidics can provide the fundamental platform technology that reduces the footprint, minimizes reagent consumption, and fully automates monitoring and analytical equipment for operation in the field, monitoring of cities, and detection in the

clinic. However, microfluidics faces many challenges before it is ready for wide-spread deployment.

The first challenge is interfacing microfluidics with the full scale samples input at the front end. 'Real world' samples may be measured in milliliter volumes while microfluidics typically manipulates nanoliter volumes: therefore the sample must be concentrated or many orders-of-magnitude of detection sensitivity will be sacrificed at the front end of the system. In addition, for biodefense and many other applications, a potentially dilute target must be detected from what can be very large volumes. The BAND program specification for civilian protection is 100 organisms in 17,000 liters of air. To work in a microfluidic system, the 'real world' input sample must therefore be reduced in volume by orders-of-magnitude while still achieving the necessary signal-to-noise to maintain sensitivity. This means taking 100s of microliters of liquids or thousands of liters of air and concentrating the input sample into nanoliters before further microfluidic processing and reactions can take place. This can be achieved by chemically, biochemically, or physically concentrating the sample.

Paramagnetic beads provide one elegant solution to both the 'macro-to-micro' interface and specificity. The beads, typically about several microns, can specifically or non-specifically capture nucleic acids, cells, viruses, or toxins, from large volumes of solution and move samples from the full volume world of milliliters into a hundreds of nanoliters. When a capture chemistry such as immunomagnetic separations or hydridization is performed with the beads, they can extract the desired target from high backgrounds and clutter. Paramagnetic beads simplify sample handling in the microfluidic world by minimizing the positioning demands on the fluidic system since magnetics can be used to recapture beads at any location and potentially eliminate diffusional losses.

Once the sample has been introduced into the submicroliter realm, the next challenge is integrating the workflow steps in a microfluidic device. As we will see, microfluidics has been applied to the individual processes and proof-of-concept publications on almost any conceivable individual step are a proof of the potential of microfluidic approaches. However, while numerous microfluidic components are well developed in academic or research settings, a key challenge for microfluidics is to either (1) fully integrate all processes to build complete 'sample-to-answer' microfluidic systems, or (2) seamlessly interface microfluidic components with each other and with 'full scale' components into a complete system. The later requires interfacing components (or modules)—such as upstream samples from

aerosol collectors, swabs, and blood—with sample preparation components and downstream analytical devices, such as real-time PCR or mass spectroscopy. Ideally best of breed modules could be interfaced once microfluidic interfaces and connections are standardized.

In any case, full process integration within a microfluidic system or module must be accomplished. The integration requires the coupling of different reactions, which may have multiple steps of sample purification, reagent addition, mixing, separation, and detection. Microvalves and micropumps are invaluable to isolate processes or reactions as individual steps and to move fluids to integrate different steps into a workflow.

The fourth challenge is designing manufacturable biodefense microfluidic systems. The system should be lower in costs to build and operate, preferably by an order-of-magnitue. The 'valley of death' from proof of concept to product must be crossed and scalable manufacturing capabilities must be implemented with only low volumes as early adopters provide the initial orders. The commercial challenge of crossing the valley of death without prior governmental commitment is substantial.

The focus of the remainder of this review is on the microfluidic technologies that can provide advanced rapid detection and identification of bioagents. We review microfluidic technologies that may be used with an emphasis on critical battlefield needs and civilian biodefense. In each section, the technology is briefly introduced, how microfluidics is being applied to advance the state of the art is reviewed, and how this is being applied to biodefense is described. In the final sections, fully integrated microfluidics systems are assessed and some of the devices under development for biodefense are presented. Other chapters in this book review the general state of microfluidics and many of the different formats having components that might be applicable to biodefense.

10.5 Microscale Sample Preparation Methods

Starting from the sample, microfluidics can be applied to lyse organisms, concentrate, pre-separate, and purify components for further processing. State-of-the-art rapid, automated, miniaturized, modular universal sample preparation systems are required to prepare nucleic acids and proteins from biological samples in order to detect and identify high priority bioagents.

This section describes some of the technologies that can be drawn upon to create a front-end that interacts with 'real world, full volume' samples.

10.5.1 Spore disruption

Analysis of intracellular nucleic acids or proteins requires that cells be disrupted by physical (sonication, heating, or bead beating) or chemical means. The most challenging biothreat organisms to disrupt are the Gram positive bacterial spores, *Bacillus anthracis* and *Clostridium botulinum*. Sonication and bead beating are the most common ways to disrupt spores today.

Microfluidic-based cell disruption using sonication has been reported. Belgrader and coworkers from LLNL reported in 1999 the development of a mini-sonication device that disrupted spores in 30 s; when coupled with a mini-chip PCR instrument, the complete analysis took 30 min [38]. This work was further extended at Cepheid where spores were lysed by a sonication horn (in conjunction with glass beads) through a flexible interface using a pressurized microfluidics cartridge [39] then the sonicated lysate was PCR amplified after reagent addition in a disposable cartridge [40]. Marentis et al. developed a piezoelectric microfluidic mini-sonicator and determined that it could lyse eukaryotic cells and spores with an efficiency of 50% lysis of *B. subtilis* spores in 30 s [41].

Laser induced disruption in a polydimethylsiloxane (PDMS) microchip has been reported for *B. atrophaeus* spores using a laser absorbing matrix at fluencies below 20 mJ/cm^2 and without matrix above that level [42]. Small laser diodes and carboxyl-terminated magnetic beads have been shown to disrupt *E. coli*, Gram-positive vegetative bacteria, and hepatitis B virus mixed with human serum in a microchip with real time detection [43].

10.5.2 Pre-separations

Following lysis, the cellular material often requires separation into component fractions. Beads, gels, and membranes can also be incorporated to perform pre-separations to remove inhibitors or concentrate samples. They can provide high mass transfer rates and be made from polymer, silica, or other substrates for microchip liquid chromatography and electrochromatography applications. Agilent has developed a commercial polymer microfluidic chip for HPLC separation using ablation of polyimide to form channels [44]. A microfluidic technique which is increasingly applied is

the use of monoliths as a stationary phase (reviewed in [45-47]). The monoliths can be made with a variety of surface chemistries, pore sizes, and functionalized coatings.

Dielectrophoretic separations can also be performed on microdevices [48]; in this regard, the Nanogen digital array will be discussed in the Microarray Section. Microchips have used dielectrophoretic separations to separate cells and nuclei with on-chip micropumps [49], and to separate erythrocytes [50]. Dielectrophoretic separations can be combined with microbeads to increase the local concentration for enhanced bead binding [51]; with microfluidic flow cytometry to sort cells [52]; with immunocapture to assay components [53]; and with ultrasonic standing waves [54]. In the future, dielectrophoresis may be applied to separate cells from debris and environmental contaminants for biodefense purifications.

10.5.3 Nucleic acid purifications

Biodefense samples are derived from a wide variety of substrates and matrices. The matrix may contain complex mixtures including inhibitory compounds (*e.g.*, mold, hemes, indigo, humic and fulvic acids, chelating agents, DNases, RNAses, and proteases) that interfere with DNA amplification, the gold standard for bioagent identification. The DoD Critical Reagents Program now provides standardized test kits composed of commonly encountered inhibitory compounds to aid biodefense development and testing.

A number of approaches have been taken to purify nucleic acids before analysis at a full volume. Early work showed that dilution of the sample can relieve inhibition contained in soil extracts for PCR amplification reactions [55]. However, dilution may not be an option if target concentrations are low. Low-melting-temperature agarose has been used to extract DNA from soil samples [56]. Solid phase extraction that adsorb analytes onto columns, beads, and surfaces and spun separation gels in column format are commonly used to purify DNA before analysis. Multistep purifications such as organic extracts combined with Sephadex columns have also been developed. While these methods are effective, they were best suited for research laboratory environments due to their reliance on supplementary equipment, trained personnel, and time-consuming procedures [57]. Most are not amenable to a microfluidic format.

Microfluidics and microchips are now being applied to miniaturize DNA extractions and concentrate nucleic acids. The surfaces of capillaries [58], beads [59], and microchips have been used with chaotrophic agents to concentrate, and purify DNA in microsystems. Microchips with silicon pillars [60-61], or plastic microchips with silicon dioxide coatings [62] can purify DNA using chaotrophic agents, ethanol wash, and elution in water. Cepheid reported nucleic acid extraction efficiencies of about 50% and concentrated samples about ten-fold for PCR [61]. Silicon microfluidic channels have been modified with amino silanes and DNA selectively eluted with alkaline rinses [63]. Immobilized beads in microchannels increased the extraction efficiency from serum 88-fold compared with free beads [64]. An integrated microfluidic device was developed to pre-treat whole blood samples using a micro-filter, micro-mixer, micro-pillar array, micro-weir and porous matrix [65]. Sol gels in capillary chromatography have been reviewed [66], as have monoliths for preconcentration and sample extraction [67].

The Landers group reported silica bead purification of DNA with chaotrophic agents and sol gel immobilization [68] on microchips, and demonstrated purification of *B. anthracis* DNA [69]. They have demonstrated a silica-based monolithic column in a fused-silica capillary [70] and on a glass microchip [71] with extraction of model DNA from complex samples with efficiencies of 70%. For whole blood and other mixtures they combined a C18 reverse phase column, to remove proteins and other compounds, with a monolithic column to create a dual phase sample preparation microchip [72-73].

The authors of this chapter have been developing a device, BeadStorm™, for automated magnetic bead purification technology on microfluidic handling on microchips (Fig. 10.1) at Microchip Biotechnologies Inc. The plastic sample processing cube, about 1 in^3, with a 800 uL processing chamber is integrated with pneumatically actuated microvalves on a glass microchip to direct pressure-driven flows consisting of fluids, beads, and samples among reagent and reaction reservoirs. Microvalves replace both conventional valves and tubing between reservoirs providing a leak-free, self-contained fluid transport. The BeadStorm module manipulates input liquid and swabs of biological samples by bead purifying the samples, fully preparing them for downstream analysis such as PCR and immunoassays. Immunomagnetic separations have also been performed in this format. The BeadStorm device has been successfully used to automate DNA extraction from buccal swabs for STR amplification. Liquid blood samples have also been successfully prepared by the BeadStorm module

with automated preparation of DNA from liquid blood sample in less than 5 minutes.

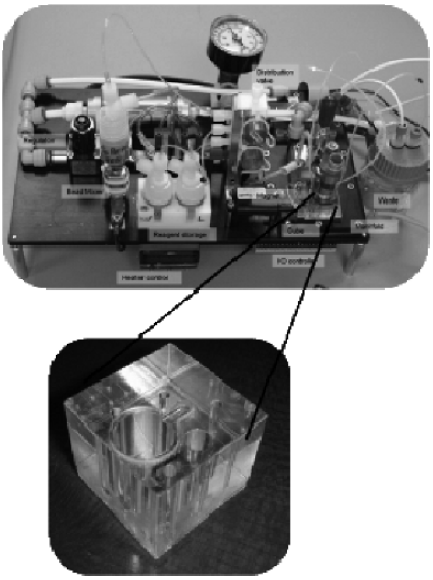

Fig. 10.1 BeadStorm device (top panel) and plastic disposable 'cube' containing a glass chip with microvalves on the bottom to direct flows (bottom panel).

10.6 Immunomagnetic Separations and Immunoassays

Immunological techniques are widely used for rapid purification and detection of cells, viruses, and proteins and are the most widely used diagnostic method in clinical medicine. The primary antibody is typically attached to a solid surface such a microtiter plate or a bead. The secondary antibody is added to generate a 'sandwich' assay that can provide a highly specific and rapid readout. The antibodies can be polyclonal, with wide variation of specificity from batch-to-batch, or monoclonal, which can be produced repeatedly with identical avidity to the corresponding antigen. Sandwich assays are well developed for use in laboratories, clinics, and field applications, and are the most common type of assays for toxin testing. There are many immunoassays that are being applied to biodefense and microfluidic immunoassays will be increasingly important in the future.

10.6.1 Immunomagnetic separations

Immunomagnetic separation (IMS) is a powerful technology that allows targets to be captured and concentrated in a single step using a mechanically simplified format that employs paramagnetic beads and a magnetic field (reviewed in [74-76]). IMS is used to capture, concentrate, and then purify specific target antigens, proteins, toxins, nucleic acids, cells, and spores in a single step. IMS works by binding a specific affinity reagent, typically an antibody, to paramagnetic beads, which are only magnetic in the presence of an external magnetic field. The beads can be added to complex samples such as aerosols, liquids, bodily fluids, or food. After binding of the target to the affinity reagent (which itself is bound to the paramagnetic bead) the bead is captured by application of a magnetic field. Unbound or loosely bound material is removed by washing purifing the target from other, unwanted materials in the original sample. A similar approach can purify nucleic acids using a complementary nucleic acid strand attached to a bead. Because beads are small and bind high levels of target, when the beads are concentrated by magnetic force, they form bead beds measured in the hundreds of nanoliters (or as low as a single bead), thus concentrating the target at the same time it is purified. The purified and concentrated targets can be conveniently transported, denatured, lysed or analyzed on-bead, or eluted off-bead for further sample preparation and analysis. Immunomagnetic separations are commonly used as an upstream purification step before qPCR, electrochemiluminescence, and magnetic force discrimination.

As mentioned, paramagnetic beads provide an excellent solution to the macroscale-to-microscale interface: beads are an almost ideal vehicle to purify samples at the macroscale from large volumes and concentrate the specific biomolecules or targets to the nanoscale for introduction into microfluidics devices.

Immunomagnetic separations are used widely for the detection of microorganisms in food and agriculture. Typically, immunomagnetic beads coated with the appropriate antibody are added to material that had been homogenized in a stomacher. The pathogenic strain *Escherichia coli* O157:H7 can be detected directly in ground beef with greater sensitivity than traditional plate enumeration methods by using IMS before plating [77]. IMS has also been coupled with qPCR [78], fluorescence microscopy, and solid-phase laser scanning cytometry [79]. Key parameters affecting detection sensitivity were shown to include the amount of nanoparticles per assay, immunoreaction incubation time, concentration of the target organism, matrix

background, and interferents. Generally, interference by non-target microorganisms is less than 1% [80]. IMS can also be combined with PCR to effectively increase the specificity of the overall process by combining the specificity of antibody-antigen recognition and the sensitivity of PCR. Immuno-quantitative PCR for *S. aureus* enterotoxin B (SEB) detection was found to be 1,000 times more sensitive than enzyme linked immunosorbent assays (ELISA), with little cross-reactivity [81].

Immunomagnetic separations have been adapted to microfluidic devices. In one application, electromagnetic gradients were generated using Cu micro-coil arrays embedded in a silicon substrate with magnetic pillars composed of NiCoP alloy with an integrated sensing coil to produce tunable, localized magnetic forces that were able to trap up to 80% of applied particles [82]. A miniature, integrated microfluidic device to separate magnetic particles from laminar flows was developed by the Whitesides group and demonstrated to be effective in separating live *E. coli* on magnetic beads [83]. Clinical applications development includes capture of targeted T cells from blood in bead beds contained in microfluidic channels. These studies resulted in 20-37% T cell capture, but only allowed flow rates of 3 uL/min [84]. Rates of hundreds of microliters per minute are required for most applications that can be achieved by increasing flow rates or by upstream deployment of IMS or other technologies that specifically capture and concentrate targets.

10.6.2 Immunoassays

In addition to performing IMS purifications, extensive work has been done on developing and integrating complete immunoassays in microfluidic devices. Immunoassays include immunochromatographic lateral flow devices, ELISA, IMS-electrochemiluminescence, time-resolved fluorescence, and magnetic force assays. In addition to widespread clinical applications, immunoassays are routinely used to detect biosecurity threats [85-87]. Future immunoassays will continue to exploit advances in antibody production and screening, miniaturization, integration, and multiplexing [88].

10.6.2.1 Lateral flow

Lateral flow assays are very simple and compatible with portable applications. A drop of test solution in buffer is added to a pad containing antibodies coupled to colloidal gold or other labels. The antibody and antigen,

if present, bind and wick down the pad laterally and intercept detection lines that contain capture antibody-gold complexes. The gold aggregates due to the bivalency of the antibodies and produces a line that is visible by eye. Home pregnancy tests are probably the best-known lateral flow devices. Lateral flow devices are simple to use, require little training or equipment, and have been devised to detect biothreat agents. Simple dipstick immunoassays for *E. coli* O157:H7 can detect 1 cell per g in ground beef, after outgrowth of the sample [89]. A comparison of lateral flow assays in a handheld device with ELISAs and PCR found the sensitivity of the lateral flow assays were approximately one-hundredth of ELISAs, which were in turn one-tenth the sensitivity of PCR assays [90].

Lateral flow devices are being improved with microfluidic technologies. Monolithic beds have been combined with electrophoretic separations to produce a fast (<10 min), sensitive assay for saliva analysis [91]. An ELISA detected staphylococcal enterotoxin B (SEB) in a handheld assay for food at about 50 pg/g of matrix using lateral flow [92]. An integrated microfluidic device with sample preparation (filtration and mixing) has been described to detect botulinum neurotoxin directly from whole blood [93].

10.6.2.2 ELISA

ELISA is the most commonly used form of immunoassay. ELISAs use an antibody bound to a solid phase support, such as a microtiter plate, to capture analytes from liquids. After washing away unbound material, a secondary antibody with a label or coupled to an enzyme (*e.g.*, horseradish peroxidase) is used to produce a visual or fluorescent readout. ELISAs are relatively inexpensive, scalable to 96- and 384-well technologies, and can be sensitive and specific, depending on the antibody pairs.

Early work with polyclonal antibodies demonstrated detection of the enterohemorrhagic *E. coli* O157:H7 in food at about 1 cell/g sensitivity [94]. Anti-*S. aureus* enterotoxin B (SEB) antibody has been immobilized on carboxylated polystyrene microparticles and a competitive assay between FITC-labeled SEB was developed to detect 0.125 ng/mL of SEB in drinking water and 0.5 ng/mL in whole milk [95]. SEB has also been assayed by direct labeling of secondary antibody with detection limits of 100 pg/well [96-97].

Immunoassays are being adapted to microfluidic devices. Microfluidic molded silicone integrated devices have successfully detected botulinum

neurotoxin serotype A with results equivalent to full volume assays [98]. Microfluidic immunoassays in plastic devices offer the affordability of plastics, the availability of diverse microfabrication methods, and many well-developed polymer surface modifications [99]. A heterogeneous immunoassay with antigens immobilized on PDMS-coated glass microchips with electrokinetic-control for multiple analyte detection had detection limits for *E. coli* O157:H7 of 3 ug/mL in an automated prototype [100]. A poly(methyl methacrylate) (PMMA) microfluidic immunoassay device was modified with poly(ethyleneimine) (PEI), an amine-bearing polymer, to increase antibody binding ten-fold [101]; the authors believe this is due to the spacer effect as well as the addition of amine groups. Due to the smaller dimensions, the microchip reactions were ten-fold faster than 96 well plates and had a dynamic range of 5 to 1000 ng/mL. An early study using glass capillary tubes as a solid support to assay *E. coli* O157:H7 employed a competitive-based immunoassay and achieved a detectable limit of 1 cfu per 10 g of ground beef [102]. Capillaries have also been employed to separate complex matrices and detect a model antigen at 10 pM with immunoaffinity chromatography using dual syringe pumps, a silica bead packed bed, and laser-induced fluorescence [103]. Beads have been used on microchips to separate binding steps from the secondary detection to reduce background [104] and to mix immunoassays on microchips [105].

Microfluidic immunoassays are being adapted for biodefense needs. Liu and coworkers [106] developed multi-stage integrated microfluidics for immunoassays utilizing electrochemical detection. Micropumps and circuits were integrated to perform parallel immunoassays for model organisms with enzyme-generated signal detected by active CMOS circuitry with resulting sensitivity in the fM range [106]. A Multi-Analyte Array Biosensor (MAAB) was developed at the Naval Research Laboratory (NRL) to detect multiple target agents in complex samples using a novel fluidics cube module to control the flow of solutions over six different immunoarray sensors in a small portable device with an evanescent wave detector [107]. The MAAB could rapidly detect three toxins: ricin, staphylococcal enterotoxin B, and cholera toxin [108], and *S. typhimurium* [109]. Liquid array-based immunoassays with multiplexed detection have also been developed and tested for model organisms [110]. Yang and coworkers at Nanogen exploited the unique characteristics of their addressable arrays to develop a device that performed automated electric-field-driven immunocapture and DNA hybridization [111].

10.6.2.3 Electrochemiluminescence assays

Immunomagnetic separations have been combined with electrochemiluminescence (ECL) detection [85]. ECL-based assays contain ruthenium labels, which emit light when electrochemically reduced. In ECL detection, tripropylamine can be oxided at the surface of an array of electrodes, and in turn it reduces the ruthenium, which then emits light. Background noise is reduced since the reaction activation is localized and controlled by the electrode. Several commercial systems are available, and over 50 immunoassays are available on one clinically aimed system. ECL has been further developed with microelectrodes manufactured using screen-printing of carbon inks onto microtiter plates.

For biodefense, work in 1995 showed that the ECL detection of biotoxoids and 100 *Bacillus anthracis* spores in less than one hour [112]. An ECL assay was compared with fluorogenic chemiluminescence (FCL) for the detection of biological threat agents [113]. SEB at a concentration of 1 pg/mL has been detected in a range of matrices using ECL in a 30 min immunoassay and found to be significantly better than ELISA reactions [114]. *Clostridium botulinum* toxins A, B, E, and F were detected at about 100 pg/mL in clinical samples and food using IMS ECL detection, about the same sensitivity as ELISA, but with much more rapid time to results [115]. ECL immunoassays have also been used to detect ricin at 0.1 ng/mL [116]. In the future, ECL detection may well be integrated with microfluidics to produce fully integrated and sensitive laboratory and portable detection systems for biothreat agents.

10.6.2.4 Time-resolved fluorescence

Time resolved fluorescence uses lanthanide chelate labels that have very long fluorescent decay times and large Stokes shifts [85]. The secondary antibody can use a lanthanide such as Europium that fluoresces in an enhancer solution. The long decay time can lead to a very low background and sensitivity that are an order of magnitude better than traditional ELISAs, but with greater variability [117]. Commercial full scale systems are in use to detect biothreat agents including *Franciscella tularensis*, *Clostridium botulinum* toxin, and SEB with detection at a range of low pg/ml [118].

10.6.2.5 Magnetic force assays

Immunoassays have been integrated with magnetic detectors to produce microfluidic systems that are being applied to biodefense and other fields.

Using this approach, the strength of intermolecular interactions can be measured by the force required to disrupt a bond when the target is attached to a magnetic bead [119-120]. The magnetic bead serves as the label which can be detected by microfabricated magnetoresistive transducers on microchips. Multiple analytes can be measured in less than 15 min in an array format with sensitivity close to ELISA [121]. Model spores and viruses can be detected at about 10^5 cfu/mL and 10^7 pfu/mL, respectively, while SEB was detected at 10 ng/mL. Multiple samples can be measured simultaneously and magnetic force is tolerant of different types of analytes. Multiplexed femtomolar detection of proteins from complex mixtures has been shown in a format that may be adapted for a handheld platform for both nucleic acid hybridization assays and immunoassays [122].

10.7 Proteomic Approaches

Proteomic approaches for biodefense rely on identification of proteins and peptides to evaluate and characterize potential biothreat agents. Primary proteomic approaches for biodefense include separation of proteins and peptides by mass spectrometry platforms [123], two-dimensional gel analysis [124], and protein arrays. Proteomic approaches are being used to build a cyberinfrastructure of NIAID-funded centers that are applying these tools for biodefense to develop vaccines and proteomic targets [125]. Data from mass spectrometry, yeast two-hybrid (Y2H), gene expression profiles, X-ray and NMR for *Bacillus*, *Brucella*, *Cryptosporidium*, *Salmonella*, SARS, Toxoplasma, *Vibrio* and *Yersinia*, human tissue libraries, and mouse macrophages have been developed [126].

Microfluidics is being widely adapted to proteomic systems as upstream devices and nozzle systems for mass spectroscopy, as reviewed in [127-128]. In essence, microfluidics is compatible with the low flow rates, small sample volumes, and microseparations that are required for mass spectrometry. Both microfabricated nozzles and HPLC devices are presently commercially available. The various developments are beyond the scope of this review, but are described in Chapter 3 of this book.

For biodefense applications, protein profiling has been developed at Sandia National Laboratory into an autonomous microfluidics system combining microfluidics sample preparation modules with microchip gel electrophoresis [129]. This system is fully automated, with a total 10 min sample

preparation and detection time. Sensitivity for *B. subtilis* spores was 16 agent-containing particles per liter of air.

Protein arrays [130] containing either antibodies to different epitopes or to different proteins arrayed on a solid surface, have been applied to characterize and type biothreats. A protein chip for the ArrayTube platform was developed that uses a microtube-integrated protein chip that accomplishes detection using the classical sandwich assay and horseradish peroxidase colorimetric substrate. Immunoassays were developed for SEB, ricin, Venezuelan equine encephalitis virus, St. Louis encephalitis virus, West Nile virus, Yellow fever virus, Orthopox virus species, *Franciscella tularensis*, *Yersinia pestis, Brucella melitensis, Burkholderia mallei* and *Escherichia coli* O157:H7 [131]. Invitrogen has been developing its high-density protein microarrays (ProtoArrays™) for detection of plague, smallpox, anthrax, and a number of hemorrhagic diseases, such as ebola and dengue fever. Phage display, where the probes on the array are selected from billions of clones, is a potentially powerful application of protein arrays and may be adopted for biodefense [132], as are peptide arrays.

10.8 Nucleic Acid Amplification and Detection Methods

For nucleic acids following sample preparation, amplification technologies can be applied that greatly increase the signal. The best known and most used DNA amplification method is PCR [133]. PCR uses thermal cycling to exponentially amplify DNA using a thermally stable DNA polymerase. Each cycle of amplification doubles the amount of template, thereby exponentially increasing the amount of target, and can amplify from as little as a single copy of DNA. The specificity of the amplification is determined by the pair of primers that initiate the amplification. PCR has become a standard clinical and research technique for nucleic acid testing (reviewed in [134-136]) and for biodefense [137]. Many different variations of the basic PCR reaction have been developed, including qPCR, nested PCR, multiplexed PCR, and single nucleotide polymorphism PCR.

qPCR has revolutionized the detection of specific DNA sequences in the laboratory, clinic, and field (reviewed in [138-139]). qPCR quantifies the amount of original target sequence using a fluorescently-labeled probe that acts as a reporter and detection system. In one version, qPCR employs a fluorescently–labeled probe that contains a quencher that suppresses the fluorescent signal. During the replication process, the signal becomes un-

quenched, emitting light. As the amplification progresses, the fluorescent signal increases. The presence and amount of target DNA in the sample can be determined from the cycle number when the signal increases over a threshold value, commonly called the cycle threshold (C_T). Multiplexed reactions make it possible to detect multiple agents in each reaction vessel. The amount of DNA produced can also be measured non-specifically using DNA intercalating dyes and other (specific) probes such as molecular beacons. qPCR has specificity and sensitivity equivalent to PCR, while simultaneously amplifying, detecting, and quantifying the original DNA target in a single, contained reaction.

10.8.1 PCR and qPCR detection of pathogens for biodefense

PCR and qPCR are the most widely used methods to detect and identify biowarfare agents by identifying target DNA sequences in the laboratory and field [140]. Multiplexed PCR assays for virulence factors on two plasmids, pXO1 and pXO2, in *B. anthracis* were able to distinguish it from closely related strains [141]. Highly specific assays can identify *B. anthracis* using pXO1, pXO2, protective antigen (*pagA),* and capsular protein B (*capB*) [142] are commercially available for clinical samples [143-144]. *B. anthracis* has been detected from soil samples at 10 spores per mL [145], in aerosols [146], and in food [147]. qPCR detection assays have been multiplexed to detect four biothreat agents, *Y. pestis, F. tularensis, B. anthracis* and *B. mallei,* simultaneously using molecular beacons complementary to conserved 16S rRNA targets [148], and with minor groove binding probes with a sensitivity of 1 fg of target with no cross reactivity [149]. Melting point curves combined with the multiplex amplification helped distinguish *B. anthracis* from *Y. pestis* and *Leishmania* [150], and between members of the *B. cereus* group [151].

The performance of three commonly used qPCR instruments was compared and generally comparable limits of detection, sensitivity, and specificity were found [152]. The GeneXpert system (Cepheid) is a standard instrument for semi- automated sample preparation and qPCR (using the SmartCycler as the qPCR platform). Its performance has been evaluated and the incorporation of the nucleic acid purification component of the sample preparation has been credited with its 1,000-fold improved detection over the SmartCycler alone for *B. anthracis* Sterne spores due to removal of inhibitors and concentration of the sample [153].

10.8.2 Miniaturized and Microfluidic PCR

Miniaturized PCR has been an area of intense development with devices developed that use microchips and capillaries to perform thermal cycling in stationary formats and continuous flow regimes. The advantages of miniaturized PCR devices are more rapid assay times, low reagent consumption, and potential integration with upstream sample preparation modules. Miniaturization of PCR, including discussion on the types of common designs, issues with surface chemistries that can inhibit PCR, coating procedures, and heating strategies has been reviewed [154-155].

Miniaturized PCR was initially performed in glass capillary tubing. Capillaries are off-the-shelf items that are suitable for sub-microliter reactions. Wittver first demonstrated (in 1989) PCR amplification of DNA in sealed capillary tubes using a hot air cycler [156]. This basic design became the Rapid Cycler (Idaho Technologies), the forerunner to RAZOR, in use today for biodefense applications. Sample volumes were reduced to 1-10 uL with cycling times of less than 15 min [157]. A medium-throughput automated capillary sample preparation system that processed 1,000 one uL samples per day was developed [158]; the Acapella-1K (U. Washington, Seattle) utilized a mechanical capillary handling system, air cycling, and a piezoelectric reagent dispenser [159]. Jovanovich and co-workers developed a 500 nL sample preparation system also using an air cycler with a 384-channel capillary cassette (Fig. 10.2). This system used 150 um ID capillaries without a coating and standard PCR conditions (except for elevated Mg^{+2} concentrations). DNA samples were forced onto the surface of the glass capillary using chaotrophic agents, the DNA-coated capillary was then evacuated, and 500 nL of PCR reagents re-filled the tube by capillary action. PCR reaction efficiencies were equivalent to full volume reactions. Capillaries have many applications in microfluidics but are currently more difficult to integrate with other functions than microchips. The integration requires robust microfluidic connectors.

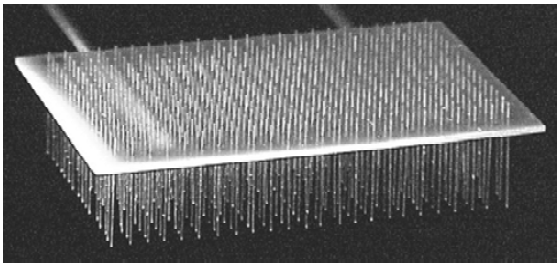

Fig. 10.2 Capillary cassette with 384 capillaries, each with a volume of 500 nL.

The first miniaturized PCR reactions in a microchip were performed in an etched silicon wafer by Northrup and coworkers, reported in 1993 [160]. Wilding *et al.* reported successful cycling in 5 and 10 uL silicon reactors cycled by an external Peltier device in 1994 [161]. PCR reactions have been performed in 87 nL in silicon devices [162], which was expanded on by Belgrader *et al*. by addition of capillary electrophoresis on microchip [163]. Belgrader and coworkers further reported PCR amplification in seven minutes [164].

The first miniaturized thermal cycler with real time detection was from Northrup and colleagues at LLNL and used silicon based reaction chambers with diode detectors and integrated heaters [165], this same group devised a portable battery-powered unit [166]. Many other reports of qPCR devices have followed [167-169].

Some of the challenges of microfluidic qPCR are control of liquid positioning, bubble formation, sealing chambers using microfluidic valves, and surface interactions. Liquids and targets can be manipulated by precise positioning by micropumps, by capture onto a surface, or by exploiting beads and solid phase chemistries. Microvalves can restrict the liquid solutions to proper chambers. One group has simultaneously sealed the reaction chamber with valves that serve as the macro-to-micro interface [170]. For poly(cyclic olefin) plastic fabrication, gel valves were used to confine amplicons before integrated on-chip gel electrophoresis [171]. Bubble formation has been found to be primarily due to surface wetting properties of the chamber [172]. Surface interactions can be minimized by improving the surface chemistry or by increasing channel dimensions to over 125 microns.

10.8.3 Heating and cooling approaches

A challenge for microfluidics is improving heating and cooling rates while maintaining good uniformity. One approach has been to microfabricate resistive heaters and sensors directly onto microchips. This has been used to achieve 20^{o} C/s heating and 10^{o} C/s cooling rates to detect upper respiratory tract infection microorganisms in 15 min [173]. A second approach, pioneered by the Landers group, used infrared heating to rapidly heat water in fluidic channels [174-175]; cycle times as low as 17s were achieved with successful PCR amplification and cycle sequencing. IR driven PCR was integrated on a microchip with upstream solid-phase extraction of DNA using silica beads in sol gel to isolate DNA from an anthrax spore-

spiked nasal swab; amplified target DNA sequences were detected on-chip with a total processing time of 25 min [176].

A third approach is flowthrough devices with fixed-temperature zones. Control of temperature and uniformity is simplified, and power consumption is low since the zones stay at a constant temperature. An added advantage is that cycling times can be reduced due to the elimination of temperature ramping. The challenge to this approach is to reduce surface interactions which can degrade PCR reaction performance. This can be accomplished using surface coatings, by controlling surface to volume ratios, or by using emulsions. Soper's group has built flowthrough devices combining flowthrough PCR in a polycarbonate chip and a PMMA chip using detection with a ligase assay [177]. Reverse transcription and PCR have been integrated in a flow through glass microchip with 55 um channels integrating different temperature zones; 30 cycles of PCR amplification were achieved in 6 min [178]. A ferrofluidic actuator has also been used to move a PCR sample plug rapidly between temperature zones [179]. A hybrid chip with silicon and PMMA has performed high speed PCR with three heated zones [180].

PCR amplification in small volumes has also been accomplished in microarray formats. The target DNA is spotted onto a glass slide that has covalently attached primers, enabling amplification of bacterial target DNA [181]. This approach has been applied to identify bacteria using amplification of rDNA sequences [182]. The Solexa DNA sequencer uses a similar process to amplify clusters of PCR products from a single template molecule on a surface.

10.8.4 Miniaturized PCR and qPCR for biodefense

One of the first miniaturized real-time PCR instruments was developed for biodefense applications. The Advanced Nucleic Acid Analyzer (ANAA), devised at LLNL, used an array of 10 silicon reaction chambers with thin-film resistive heaters and solid-state optics to rapidly test samples for simulants of biothreat agents with detection limits of 100 to 1,000/mL [183-184]. A compact version of a qPCR instrument with a notebook computer, two reaction modules with integrated four-color fluorescence detection was developed and shown to detect *Bacillus* spores [166]. A handheld device, the Handheld Advanced Nucleic Acid Analyzer (HANAA), also developed at LLNL, used plastic reaction tubes with silicon and platinum-based thermal cycler units and two light emitting diodes

to detect bacteria including *B. anthracis* Ames [185]. The LLNL group, working with Sandia, went on to develop the APDS, which performs continuous monitoring with a multiplexed immunoassay trigger and confirmatory qPCR assays. This has been demonstrated for aerosolized *B. anthracis*, *Yersinia pestis*, *Bacillus globigii,* and botulinum toxin [186]. LLNL and Sandia also developed a "Biobriefcase" device with a smaller footprint that has multiplexed, autonomous detection with immunoassays, toxin assays, and qPCR. The Biobriefcase incorporated inhibitor removal and concentrated the sample by mixing aerosol-collected liquid with a chaotrophic agent prior to silica bead bed purification [187]. The LLNL group has also developed a 10-plex PCR amplification with hybridization to beads upstream of a flow cytometry readout; 1000 samples were processed in 8 hours [188]. The LightCycler (Idaho Technology) has been adapted into a "ruggedized" Advanced Pathogen Identification Device, RAPID, which has been used for field analysis of bioagents and pathogens [189]. *Y. pestis* was detected at about 20 genome equivalents in 75 min using this system. The latest innovation from LLNL is amplification in emulsion of single copy DNA in a lab-on-a-chip format; the system produces 10 pL droplets that can perform qPCR with thresholds exceeded significantly earlier than conventional instruments [190].

In addition to detecting DNA targets, biodefense needs require that RNA viruses be detected. Detection of two RNA-based viruses—Dengue virus and enterovirus 71 has been demonstrated with a PDMS microchip that integrates reverse transcriptase and PCR amplification [191]. Parallel reverse transcriptase-PCR assays have been performed in 450 pL and shown to detect as little as 34 copies [192]. A fully integrated handheld device using isothermal nucleic acid sequence-based amplification (NASBA) [193], which specifically amplifies RNA from primers, was built and shown to have comparable results to laboratory instruments.

10.8.5 Other Nucleic acid amplification methods

In addition to PCR there are a number of other nucleic acid amplification technologies that are being adapted for biodefense: strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), exponential amplification reaction (EXPAR), and rolling circle amplification (RCA). SDA uses the primer-directed nicking activity of a restriction enzyme and the strand displacement activity of an exonuclease-deficient polymerase to amplify DNA [194]. SDA can achieve 10^8 to 10^{10} amplification in about 15 min [195]. Fluorescence resonance energy transfer

(FRET) probes can be incorporated and real time instruments for the clinical laboratory are in commercial use [196-197].

EXPAR is a DNA amplification reaction that produces short (8-22 nucleotides) in a linear or exponential amplification in isothermal homogeneous assays [198]. EXPAR can be extremely rapid with amplification of greater than 10^6. Both real time [199] and end point formats have been developed. EXPAR has been applied to biodefense applications as part of the BAND project and in other projects as a quick, specific amplification technique.

LAMP is an isothermal DNA amplification method that relies on autocycling strand-displacement DNA synthesis. Four sets of primers are used with the thermostable *Bacillus stearothermophilus* (BST) DNA polymerase that has high strand displacement activity [200]. The reaction produces a white precipitate, magnesium pyrophosphate, which can be easily detected as an indicator of a positive amplification reaction [201]. The specificity and sensitivity are competitive with PCR, and in some cases more sensitive than nested PCR [202].

RCA is an isothermal method that uses Phi29 DNA polymerase to amplify circular DNA [203]. Phi29 DNA polymerase is a single subunit with excellent processivity and can amplify $>10^7$ fold [204]. RCA is widely used for whole genome amplification, for scarce material, and can nonspecifically amplify trace amounts of DNA. RCA is routinely used at genome centers preparing template for sample preparation for DNA sequencing [205]. RCA has also been used for cell free cloning of genomic DNA that might be lethal to cells [206]. RCA is a powerful tool for forensic and biodefense applications.

10.9 Microarrays

DNA microarrays have many potential applications in biodefense. DNA microarray technology is a widely used powerful technique that uses large arrays of microspots of DNA on a solid support or beads to detect complementary DNA or RNA products from a sample. (Protein arrays were briefly discussed in the Proteomics section). For RNA samples, amplification of RNA is commonly done by first performing reverse transcriptase to create DNA from RNA, and then the resulting DNA can be amplified using standard DNA amplification methods such as PCR, or whole genome amplification, or by in vitro transcription followed by another cycle of re-

verse transcriptase. Fluorescent labeling can be introduced into the sample at various steps in the process. Typically, tens of thousands of spots, each containing a unique sequence, are interrogated in a single experiment with fluorescent detection. The data can represent a fingerprint of the transcriptional state of an organism (e.g. biothreat agent, or of the response of a human potentially infected with the agent), identify DNA sequences present in an organism, or resequence organisms [207-208]. The strength of the microarray platform is the depth of characterization. The 10,000s of analytes measured on a single microarray slide can generate massive amounts of data. In the future, DNA microarrays may be displaced or challenged by digital gene expression methods using next-generation DNA sequencing to produce 100,000's or more sequences from a sample or single mRNA detection and enumeration strategies (*e.g.,* Nanostring).

Microarrays have been combined with PCR amplification to identify and genetically discriminate *B. anthracis* from closely related bacterial species from the *B. cereus* group and determine if the strains harbor plasmids [209]. DNA microarrays can potentially detect multiple pathogens in a single sample. The FDA has developed a microarray, FDA-1, to screen for several food pathogens and virulence factors, including SEB [210]. SAIC developed its Phase I BAND project based upon a fully automated system that would collect air samples and then analyze them for pathogens using microarrays. After collection and filtration, reverse transcriptase and PCR amplification were used before hybridization to a DNA microarray in a cartridge for ten min. The data was then analyzed for fingerprints that indicated the presence of threat agents.

10.9.1 Microarrays and microfluidics

Microarray sample preparation is a complicated, multistep process that is dependent on the variability of individual operators. Microfluidics, with its potential to automate and integrate processes, has been applied to simplify and standardize sample preparation (reviewed in [211]). This seminal work was performed by Anderson and colleagues at Affymetrix, where a miniaturized integrated microdevice was developed to prepare samples by accessing 10 reagents and performing 60 automated operations before performing hybridizations to a microarray. Anderson and coworkers elegantly demonstrated a plastic miniaturized sample preparation system for microarray sample preparation and analysis by hybridization, and showed the detection of mutations in the HIV genome from serum samples [212]. The advantages of microfluidics for microarray biodefense applications are in-

tegration of sample preparation steps, potential reductions in reagent cost, ease of use, and decreased hybridization times.

The Soper group has integrated microarrays with microfluidic technology in plastics. A microarray was fabricated in PMMA using UV exposure of the polymer surface, coupling of amine-terminated oligonucleotide probes to the surface, and washing the surface [213]. The hybridization and allele-specific ligase detection reactions were performed in a polycarbonate flow-through biochip. The system could screen for mutations in 20 min.

Work by Liu and coworkers at Motorola and then Combimatrix has developed biochips that integrate sample preparation, PCR, and microarray detection [214]. Electrochemical pumps were used with paraffin-based single-use microvalves to regulate flow. Detection of pathogens from whole blood [214], identification of influenza virus [215], and gene expression from a cell line [216] were shown.

A microelectronic array system was developed by Nanogen for microarray testing [217]. This system employs dielectrophoresis as a sample preparation method and the hybridization of nucleic acid to probes attached to electrodes is accelerated by application of an electrical potential. The electrodes are covered with a hydrogel. Detection is performed with fluorescence probes [218].

10.10 Microelectrophoresis and Biodefense

Electrophoresis is a powerful separation technology with many biodefense applications. The advent of capillary electrophoresis (CE) and the multichannel capillary array electrophoresis (CAE) propelled the applications of electrophoresis by increasing separation speeds, automating the analysis, and improving data reliability. Most notably, CAE technology was partially responsible for the early completion of the Human Genome Project and has become the separation method of choice for most nucleic acid applications.

Several PCR-based methods for detailed laboratory characterization of microorganisms have been developed that rely on electrophoretic separation. In this section, we first review several of the separation based typing methods for bacteria identification and discrimination, describe the application of microfluidics on microchips to microelectrophoresis, and discuss

applications of DNA sequencing in a microfluidic platform to biodefense [219].

VNTR loci analysis [220] is a powerful laboratory tool for identifying bacteria, humans, and other organisms. Analogous to microsatellite genotyping, VNTR PCR amplification detects regions where genetic drift has created variable numbers of tandem repeats inserted into the genome, plasmids, or other extra-chromosomal elements. VNTR has been used to identify *Y. pestis* [221], *Mycobacterium tuberculosis* [222], and numerous other organisms.

MLVA [223] extend VNTR to assay multiple alleles and provide a fingerprint, analogous to forensics identification by microsatellite DNA analysis. Keim and co-workers have identified a set of eight VNTR regions that are diagnostic for *B. anthracis* and characterized 426 *B. anthracis* isolates into 89 distinct genotypes [223]. MLVA was used to subtype the anthrax strains from the bioterrorist attack in 2001 within eight hours of receiving isolates [224]. MLVA has been applied to type closely related *Bacillus* strains but at times required additional information for confirmatory determination of *B. anthracis* [225]. MLVA types of *Bacillus anthracis* could be further differentiated by single-nucleotide repeats [226].

AFLP is a PCR-based method that can fingerprint microbes [227], type organisms, and identify phyllogenetic relationships. AFLP is rapid—it employs a relatively simple multi-step workflow with standard reagents regardless of the organism typed. AFLP has been applied to differentiate a wide variety of microorganisms at the subspecies level [228], including *B. anthracis* [229] and is widely used to classify plants, yeasts, and other organisms. The AFLP process begins with a two-enzyme restriction digest followed by ligation of fluorescently labeled restriction half-site adapters to reconstruct the restriction site at the end of the fragments and serve as PCR primers. Two or three degenerate nucleotides on the end of the adapter reduce the complexity of the amplified products during the high stringency PCR amplification. By selection of proper restriction enzyme pairs, fluorescently labeled fragments in the range of 100 to 1,000 bases can be produced, and then are separated and detected by capillary electrophoresis. The resultant pattern is a fingerprint of the test organisms' genomic and extra-chromosomal restriction patterns. A post-labeling fluorescent method using dye-terminator chemistry can visualize both RFLP and AFLP products [230]. cDNA-AFLP can also be performed to visualize the gene expression pattern of an organism [231].

Subtle strain-to-strain variations can be characterized by single nucleotide polymorphisms (SNPs). SNP typing of intragenic spacers in the 16S-23S region has been shown to differentiate closely related *Bacillus* strains including *B. cereus* from *B. anthracis* [232]. The identification of the specific strain of a biothreat organism can help microbial forensics trace the origin and determine whether multiple incidents were caused by release of identical organisms and therefore share a common origin.

To determine strain variations, the genomic sequence can be determined by DNA sequencing. Following the September 11th attacks, the CDC sequenced the 16S rRNA gene to definitely identify *B. anthracis* from culture-negative clinical specimens of patients with confirmed anthrax [233]. In other studies, 183 16S rRNA and 74 23S rRNA sequences for all species in the *B. cereus* group showed disagreement with phenotyping clustering, but by utilizing rRNA together with *gyrB* sequences these workers could discriminate between groups [234]. Ruppitsch and coworkers showed for a wider variety of strains that sequencing of the 16S rRNA gene is not always sufficient, but that additional sequencing of intragenic sequences can increase resolution and thus differentiate bioagents [235].

10.10.1 Microelectrophoresis technologies

CAE is based upon separation in a capillary, itself a type of microdevice. In this review, we use microelectrophoresis to connote separations on microchips. The interested reader is referred to reviews [236-238] for details of chip construction, coatings, operation, and equipment. Most commonly, glass microchips are employed but plastic devices have also been developed [239].

Microchips have the potential to simultaneously separate hundreds of samples in minutes. Microchips typically consume only picoliters of samples and thus can be well matched to microscale sample preparation volumes. Fluorescently labeled amino acids [240], DNA restriction fragments [241-243], PCR products, short oligonucleotides [244], and sequencing ladders [245] have been separated by microchip capillary electrophoresis [246]. The analyses are extremely rapid, from less than a minute for oligonucleotides to less than 20 minutes for DNA sequencing [247]. We note that next generation microfluidic sequencing-by-synthesis [248], microfluidic pyrosequencing [249], sequencing by ligation, and nanopores may, in the future, have biodefense applications for detecting genetically modified organisms and digital gene expression.

Interfacing upstream microfluidic sample preparation with microscale separations is challenging. The classic Twin-T injector defines roughly a 100 to 500 picoliter volume in a short sample plug (100 to 500 um) in the separation channel with electrokinetic loading [250]. This is only a small fraction of the sample, even if the prepared sample is only 100 nL: for biodefense applications this means losing orders of magnitude of potential sensitivity during the analysis step. This can be ameliorated with isotachaphoresis, field amplified stacking, DNA binding, and other methods that locally increase the sample concentration. Isotachaphoresis concentrates samples between leading and trailing electrolytes and can increase detection limits by 50-fold or more for DNA [251-252], and stack up to a million-fold [253]. Field amplified sample stacking, routinely used in capillary electrophoresis, has been adapted to microchip electrophoresis using pressure driven flows to move low osmotic strength samples into position for stacking on the column. Concentration effects range from 65-fold increase in signals [254] to 180-fold for model compounds [255].

Separations on microchips are most developed for DNA separations. In 1998, 96 hemochromatosis samples were genotyped in less than 8 min by microelectrophoresis on a microchip [256]. Locus-specific, multiplex PCR products specific for deletions causing Duchene/Becker muscular dystrophy have been separated on a silicon-glass microchip, and T-cell receptor-gamma genes and immunoglobulin heavy chain gene on glass microchips [257]. Fluorescently-labeled CTTv PCR samples and short tandem repeats have been analyzed on single-channel CE microchips [258]. The Mathies laboratory genotyped 384 hemochromatosis samples in less than 6 min, with detection by a four-color rotary confocal fluorescence scanner [259].

DNA sequencing on microchips was first performed in 1995 in the Mathies' lab [260]; fluorescently labeled DNA sequencing fragment ladders were separated on glass microchips with a denaturing polyacrylamide sieving matrix and readlengths of about 200 bases obtained in 15 min. Mathies' lab later reported readlengths of about 500 bases in about 30 min in single channel [261]. Jovanovich's group reported the first multichannel DNA sequencing in an array of 16 microchannels with readlengths of 450 bases in 15 min in an automated instrument [247]. High throughput DNA sequencing in 96 channels obtained readlengths of 430 high quality bases [262]. To achieve longer readlengths, microchips with 40 to 50 cm separation channels were constructed and readlengths of 600 to 800 bases reported with plates containing up to 384 channels [263].

Separations on plastic microchips have also been achieved. One of the first microelectrophoresis devices, in 1993, used a twin T injector on a PDMS substrate to separate proteins and DNA [289]. Plastic substrates offer the advantage of potential low cost fabrication once high production volumes are achieved, but have been plagued by higher background fluorescence signals than glass or quartz devices and require specialized surface coatings [264]. While a detailed review is outside the scope of this article, injection molded microelectrophoresis devices have achieved separation of DNA fragments [265-266] and DNA sequencing [267]. Hot embossed PMMA with IR detection was shown to sequence DNA out to 450 bases in linear polyacrylamide [268].

For biodefense, the breakdown products of G-type and V-type nerve agents can be assayed by CE and microelectrophoresis [269]. Wang et al. [270] combined microchip electrophoresis with derivatization and electrochemical detection of thiol-containing degradation of V-type nerve agents at micromolar concentrations in less than four min. A microChemLab portable device, developed at Sandia National Laboratory, with a fused silica microfluidic separation chip, a miniature LIF detector, and high voltage power supplies [271] can assay proteins and toxins at nanomolar concentrations [272].

10.11 Integrated lab-on-a-chip systems and biodefense

The achievement of working devices for both microfluidic based sample preparation and analysis has created the possibility for complete system integration to meet biodefense needs. For robust biodefense applications, sample preparation and analysis must be integrated either directly in a monolithic format or by microfluidic connections. As described above, for DNA microarrays sample preparation has been integrated with analysis, as first shown by Anderson, and brought to commercial product by Combimatrix.

Today, one of the most advanced areas for complete system integration has been the integration of upstream sample preparation reaction steps with microelectrophoresis on microchips. Microchip and capillary-based analysis systems have the advantages of high-resolution separations with extremely fast separation times, automation, and nanoliter-scale consumption of reagents. Integration of PCR reactions in microfabricated devices with microelectrophoresis was first demonstrated in 1996 in collaboration be-

tween Northrup's LLNL group and the Mathies laboratory; PCR amplification was performed in a silicon device mounted on a glass separation device and complete analysis was achieved in 20 min for a cloned human gene and 45 min for a bacterial genomic target [273].

Early work exploited the small volumes in capillaries to prepare samples and analyze them by capillary electrophoresis. Swerdlow and colleagues developed an automated prototype with a sample loop in an air cycler coupled to a separation capillary [274]. A fully integrated miniaturized integrated microsystem using capillaries was shown to perform all steps including PCR amplification in 125 nL and separations starting from buccal cells [275]. Integration of PCR reactions in capillaries with separations in capillaries has been shown to be effective for DNA typing from blood [276] and other materials for human and viral targets [277]. PCR reactions and DNA cycle sequencing in capillaries and capillary separations have been fully integrated to create a microvolume system with readlengths of 257 bases in 4 hrs from human DNA [278].

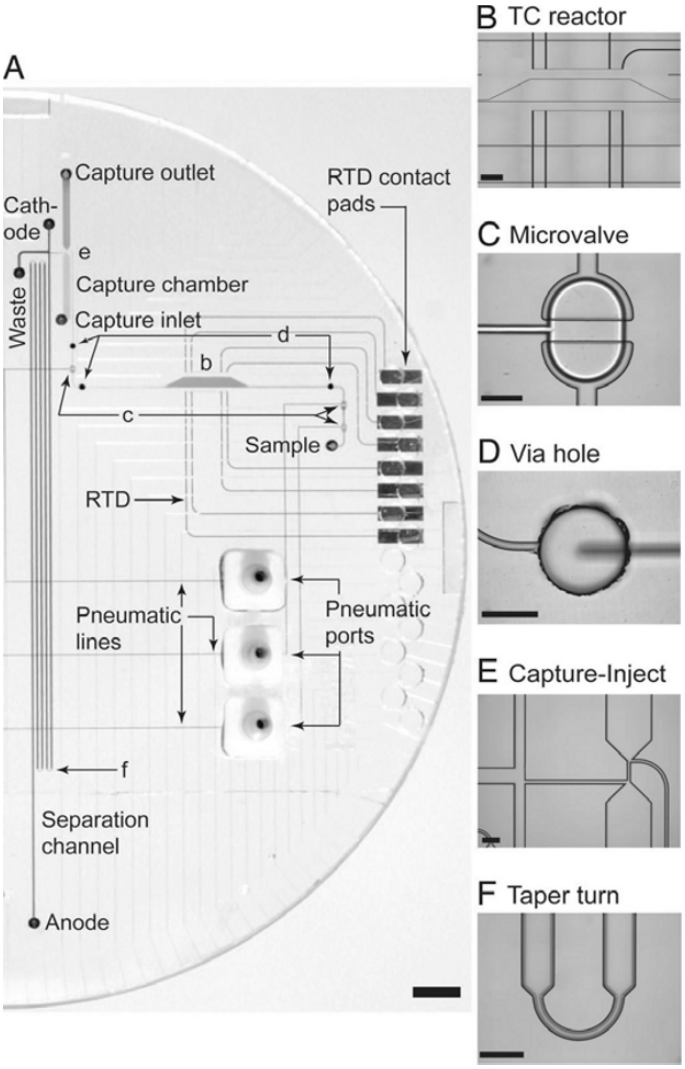

Fig. 10.3 Integrated sample preparation, clean-up, and analysis for DNA sequencing on a microchip, from [283]. Bioprocessor components. (A) Photograph of the microdevice, showing one of two complete nucleic acid processing systems. (Scale bar, 5 mm.) B–F correspond to the following component microphotographs. (B) A 250-nl thermal cycling reactor with RTDs. (Scale bar, 1 mm.) (C) A 5-nl displacement volume microvalve. (D) A 500-um-diameter via hole. (E) Capture chamber and cross injector. (F) A 65-um-wide tapered turn. (Scale bars, 300 um.) All features are etched to a depth of 30 um. Copyright (2006) National Academy of Sciences, U.S.A.

PCR reaction chambers have now been monolithically integrated with microelectrophoresis to create fully integrated microsystems that perform PCR on the same device as the separation. Burns et al. integrated sample preparation, gel electrophoresis, and on-chip detection for low resolution separation of DNA fragments [279]. The Mathies lab showed PCR amplification and microelectrophoresis separation to determine sex of humans from DNA in 15 min [280]. A portable system with 200 nL PCR reactors, solid state lasers, pneumatically actuated on-chip micropumps, and microelectrophoresis was able to detect 2-3 *E. coli* or *S. aureus* cells in less than 10 min including determining drug resistance [281]. The Mathies lab has extended their DNA sequencing on microchips upstream to include integrated nanovolume sample preparation and purification. First, nanoscale PCR reactions were combined with capillary electrophoresis analysis [282]. Then, sample preparation, cleanup, and DNA separations were combined to integrate DNA sequencing, as shown in Fig. 10.3, [283]. A 250 nL cycle sequencing reaction was performed on microchip, and then moved by micropumps onto an acrylamide gel capture matrix with an oligonucleotide hybridization probe to capture the target DNA in a 60 nL capture chamber. The affinity capture removes template DNA, desalts, and pre-concentrates the sample for microchip electrophoresis for higher injection efficiencies. The sample is then electrophoresed into an injector and separated on microchip. Readlengths up to 556 bases were obtained from 1 fmol of template. The key to the integration was pneumatically actuated microvalves and micropumps [288].

The Landers laboratory has completely integrated PCR, sample cleanup, and capillary electrophoresis on microchips, as shown in Fig. 10.4. A device with PCR reactions, IR mediated heating, pneumatically actuated on-chip micropumps, and microelectrophoresis has achieved amplification and good separations in less than 12 min [284]. They have detected of *B. anthracis* from whole blood of asymptomatic mice and *Bordetella pertussis* from nasal aspirate of a patient using on-chip nucleic acid purification with a 550 nL PCR reactor coupled to microelectrophoresis analysis with control by pneumatic microvalves [285].

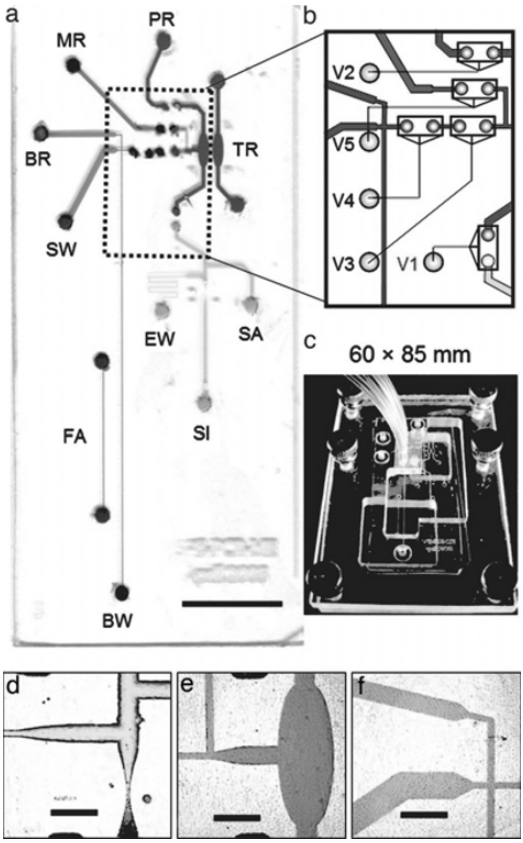

Fig. 10.4 Fully integrated device for biodefense detection, from [285]. Images of the device. (*a*) (Scale bar, 10 mm.). Domains for DNA extraction, PCR amplification, injection, and separation are connected by channels and vias. SPE reservoirs are labeled for sample inlet (SI), sidearm (SA), and extraction waste (EW). Injection reservoirs are labeled for PCR reservoir (PR), marker reservoir (MR), and sample waste (SW). Electrophoresis reservoirs are labeled for buffer reservoir (BR) and buffer waste (BW). The flow control region is outlined by a dashed box. (*b*) Schematic of flow control region. Valves are shown as open rectangles. (*c*) Device loaded into the manifold. (*d*) Intersection between SI and SA inlet channels, with the EW channel tapering to increase flow resistance. (Scale bar, 1 mm.) (*e*) Image of PCR chamber with exit channel tapering before intersecting with the MR inlet channel. (Scale bar, 1 mm.) (f) Image of cross-tee intersection. (Scale bar,1 mm.). Copyright (2006) National Academy of Sciences, U.S.A.

10.11.1 Full microfluidic integration for biodefense

Fully integrated 'industrial strength' microfluidics is being applied to Homeland Security and biodefense in the BAND program by several groups including Microfluidic Systems Inc, IQuum, and US Genomics. IQuum (www.iquum.com) is developing a Liat™ "detect-to-treat" system. The system has a disposable cassette containing reagents and equipment that contains air sampling, sample preparation, and real time detection using PCR in a "lab-in-a-tube" [286]. The sample moves through segmented tube sections containing the reagents using peristaltic pumps and moves back and forth between temperature zones to amplify the DNA.

US Genomics (www.usgenomics.com) has taken a very different approach. Single stranded DNA is prepared from aerosol samples and a set of fluorescently labeled probes added. The probes bind to their homologous targets, if present. The labeled DNA is then moved through a narrow channel one molecule at a time where it is interrogated by laser-induced fluorescence. The resulting pattern of binding sites is a fingerprint that can identify known and unknown agents from a database [287]. One advantage of the approach is the analysis of individual molecules is done without apriori knowledge of the sequence. This potentially enables detection of genetically engineered or previously unknown pathogens. In the future, next generation DNA sequencers, mass spectroscopy, and DNA microarrays will provide additional solutions to begin to address genetically engineered organisms.

10.12 Summary and Perspectives

Microfluidics will become an increasing important element of the future biodefense portfolio. As shown in this and other chapters in this volume, microfluidic components are available in many different shapes and formats: proof-of-concept of just about any imaginable type of sample extraction, lysis, pre-separations, sample preparation, assay, separation, and detection component has been demonstrated in research settings, at universities, research facilities, national laboratories, and in industry. The missing component is the integration of the many parts into complete working systems that are robust, manufacturable, and maintainable.

Complete integration of complex workflows has proved elusive in full volume devices and in microfluidics. To fully integrate a microfluidic system for biodefense requires taking a sample of several hundred microliters or

larger and processing it completely to yield an answer. The chemical and biochemical workflow usually has many steps that all have to be developed, tested, and integrated. In addition, of course, surface chemistries, temperature, and other variables must be controlled.

The work described in this review has shown the promise for microfluidics for many types of genomic and proteomic devices. Fully microfluidic integrated devices that can input samples and perform sample preparation and analysis are just beginning to appear, as are field portable microfluidic genetic analyzers. While there are some examples of success at the research and prototype levels, there are only a few fully integrated solutions and fewer commercial successes.

Two main microfluidic elements are needed for full integration. The first element is an appropriate level of control of the microfluidic volumes. This typically requires microfluidic valves, a pumping mechanism, and routers to move, mix, and split, and aliquot liquids. Single use valves enable many applications in a disposable cartridge, while programmable valves that enable multiple uses are required for environmental monitoring and reusable applictions. Pneumatically driven programmable microvalves are only now becoming more mature and are in use in academia and industry to control fluid flows and mixing. These microvalves [288] have proved invaluable in the integration by the Mathies and Landers groups, and in industry to integrate processes onto monolithic microdevices.

The second enabling element will be to connect different microfluidic devices together using standard connections. In microelectronics, USB, Firewire, and other standard connectors allow devices to interconnect by defining the interface and the protocols. Microfluidics now needs to establish standards for connections that will enable ‘best in class’ microfluidic devices to work together in a ‘plug and play’ manner. A symposium of industry and academia with government representation, including DoD, DHS, and NIST, to discuss achievable initial standards would be invaluable and may serve to begin the path towards interconnectivity for microfluidics for biodefense and other applications. In this regard, the physical dimension of spacing for connectors needs to be standardized, and standard protocols for transfer of materials developed.

The future for microfluidics will be bright as individual steps are optimized and integrated. Future microfluidic systems will connect directly to ‘real world’ samples and fully integrate upstream sample concentration and analysis in a single autonomous device. The full integration of micro-

fluidic processes will enable man-portable and then hand-held biodefense devices. Eventually, if biothreats become pervasive, microfluidic home and business security devices akin to smoke detectors may provide the massive sampling capability needed to detect to warn the public. The public and the biodefense community await the transformation that complete microfluidic integration of biodefense detection can bring to increase the biosecurity of the world.

References

1. Boscarino JA, Figley CR, Adams RE (2003) Fear of terrorism in New York after the September 11 terrorist attacks: implications for emergency mental health and preparedness. Int J Emerg Ment Health 5(4):199-209.
2. Venkatesh S, Memish ZA (2003) Bioterrorism—a new challenge for public health. Int J Antimicrob Agents 21(2):200-6.
3. Heller, MB, ML. Bunning, ME. France, DM Niemeyer, L Peruski, T Naimi, et al. (2002) Laboratory Response to Anthrax Bioterrorism, New York City, 2001. Emerg Inf Dis 8:1096-1102.
4. Bennett RL (2006) Chemical or biological terrorist attacks: an analysis of the preparedness of hospitals for managing victims affected by chemical or biological weapons of mass destruction. Int J Environ Res Public Health 3:67-75.
5. Rebmann T, Carrico R, English JF (2007) Hospital infectious disease emergency preparedness: a survey of infection control professionals. Am J Infect Control 35:25-32.
6. Alexander GC, Larkin GL, Wynia MK (2006) Physicians' preparedness for bioterrorism and other public health priorities. Acad Emerg Med 13(11):1238-41.
7. Williams, M (2006) The knowledge. Technology Review. 109:44-53.
8. United States General Accounting Office, (20000 Report to Congressional Requesters, Biological Weapons: Effort to Reduce Former Soviet Threat Offers Benefits, Poses New Risks.
9. Henderson DA (1999) The looming threat of bioterrorism. Science 283(5406):1279-82.
10. Petro JB, Carus WS (2005) Biological threat characterization research: a critical component of national biodefense. Biosecur Bioterror 3(4):295-308.
11. Cirino NM, Musser KA, Egan C (2004) Multiplex diagnostic platforms for detection of biothreat agents. Expert Rev Mol Diagn 4(6):841-57.
12. Klietmann WF, Ruoff KL (2001) Bioterrorism: implications for the clinical microbiologist. Clin Microbiol Rev. 14(2):364-81.
13. Hamilton MG, Lundy PM (2007) Medical countermeasures to WMDs: defense research for civilian and military use. Toxicology 233(1-3):8-12.
14. Russell PK (2007) Project BioShield: what it is, why it is needed, and its accomplishments so far. Clin Infect Dis 45 Suppl 1:S68-72.

15. Shea DA, Lister SA (2003) The BioWatch Program: Detection of Bioterrorism. Congressional Research Service Report No. RL 32152.
16. www.milnet.com/wh/DoHS/BioWatchFactSheetFINAL.pdf
17. Comments made in "Meeting Minutes," CDC Information Council, February 27, 2003, found online at http://www.cdc.gov/cic/minutes/CIC%20minutes%202-27-03.pdf.
18. DHS' Management of BioWatch Program, OIG-07-22, January 2007.
19. Hearing Before The Subcommittee On Technology And Innovation Committee On Science And Technology House Of Representatives One Hundred Tenth Congress First Session The "Department Of Homeland Security's R&D Budget Priorities For Fiscal Year 2008" March 8, 2007, Serial No. 110–8, 33–611PS 2007. Available via the World Wide Web: http://www.house.gov/science.
20. Koplan J (2001) CDC's strategic plan for bioterrorism preparedness and response. Public Health Rep 116 Suppl 2:9-16.
21. Ashford DA, Kaiser RM, Bales ME, Shutt K, Patrawalla A, McShan A, Tappero JW, Perkins BA, Dannenberg AL (2003) Planning against biological terrorism: lessons from outbreak investigations. Emerg Infect Dis 9:515-9.
22. Crutchley TM, Rodgers JB, Whiteside HP Jr, Vanier M, Terndrup TE (2007) Agroterrorism: where are we in the ongoing war on terrorism? J Food Prot 70:791-804.
23. Cupp OS, Walker DE 2nd, Hillison J (2004) Agroterrorism in the U.S.: key security challenge for the 21st century. Biosecur Bioterror 2:97-105.
24. Niemeyer D. JBAIDS Team. (2004) Improving Laboratory Capabilities for Biological Agent Identification. J of Homeland Security March.
25. Lim DV, Simpson JM, Kearns EA, Kramer MF (2005) Current and developing technologies for monitoring agents of bioterrorism and biowarfare. Clin Microbiol Rev 18:583-607.
26. Jannes G, De Vos D. (2006) A review of current and future molecular diagnostic tests for use in the microbiology laboratory. Methods Mol Biol 345:1-21.
27. Peruski LF Jr, Peruski AH (2003) Rapid diagnostic assays in the genomic biology era: detection and identification of infectious disease and biological weapon agents. Biotechniques 35:840-6.
28. Ivnitski D, O'Neil DJ, Gattuso A, Schlicht R, Calidonna M, Fisher R (2003) Nucleic acid approaches for detection and identification of biological warfare and infectious disease agents. Biotechniques 35:862-9.
29. Hoffmaster AR, Fitzgerald CC, Ribot E, Mayer LW, Popovic T (2002) Molecular subtyping of Bacillus anthracis and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg Infect Dis 8(10):1111-6.
30. Jones SW, Dobson ME, Francesconi SC, Schoske R, Crawford R (2005) DNA assays for detection, identification, and individualization of select agent microorganisms. Croat Med J 46:522-9.
31. Chain P, Kurtz S, Ohlebusch E, Slezak T (2003) An applications-focused review of comparative genomics tools: capabilities, limitations and future challenges. Brief Bioinform 4:105-23.

32. Slezak T, Kuczmarski T, Ott L, Torres C, Medeiros D, Smith J, Truitt B, Mulakken N, Lam M, Vitalis E, Zemla A, Zhou CE, Gardner S (2003) Comparative genomics tools applied to bioterrorism defense. Brief Bioinform. 4:133-49.
33. De BK, Bragg SL, Sanden GN, Wilson KE, Diem LA, Marston CK, Hoffmaster AR, Barnett GA, Weyant RS, Abshire TG, Ezzell JW, Popovic T (2002) A two-component direct fluorescent-antibody assay for rapid identification of Bacillus anthracis. Emerg Infect Dis 8:1060-5.
34. Belgrader P, Smith JK, Weedn VW, Northrup MA (1998) J Forensic Sciences 43:315-319.
35. Belgrader P, Benette W, Hadley D, Richards J, Stratton P, Mariella R, Milanovich F (1999) Science 284:449-450.
36. United States General Accounting Office, Report to Congressional Requesters: Bioterrorism Information Technology Strategy Could Strengthen Federal Agencies' Abilities to Respond to Public Health Emergencies, GAO-03-139, May 2003.
37. Hindson BJ, Makarewicz AJ, Setlur US, Henderer BD, McBride MT, Dzenitis JM (2005) APDS: the autonomous pathogen detection system. Biosens Bioelectron 20:1925-31.
38. Belgrader P, Hansford D, Kovacs GT, Venkateswaran K, Mariella R Jr, Milanovich F, Nasarabadi S, Okuzumi M, Pourahmadi F, Northrup MA (1999) A minisonicator to rapidly disrupt bacterial spores for DNA analysis. Anal Chem 71:4232-6.
39. Taylor MT, Belgrader P, Furman BJ, Pourahmadi F, Kovacs GT, Northrup MA (2001) Lysing bacterial spores by sonication through a flexible interface in a microfluidic system. Anal Chem 73:492-6.
40. Belgrader P, Okuzumi M, Pourahmadi F, Borkholder DA, Northrup MA (2000) A microfluidic cartridge to prepare spores for PCR analysis. Biosens Bioelectron 14:849-52.
41. Marentis TC, Kusler B, Yaralioglu GG, Liu S, Haeggström EO, Khuri-Yakub BT(2005) Microfluidic sonicator for Q- disruption of eukaryotic cells and bacterial spores for DNA analysis. Ultrasound Med Biol 31:1265-77.
42. Hofmann O, Murray K, Wilkinson AS, Cox T, Manz A (2005) Laser induced disruption of bacterial spores on a microchip. Lab Chip 5:374-7.
43. Lee JG, Cheong KH, Huh N, Kim S, Choi JW, Ko C (2006) Microchip-based one step DNA extraction and qPCR in one chamber for rapid pathogen identification. Lab Chip 6:886-95.
44. Yin H, Killeen K (2007) The fundamental aspects and applications of Agilent HPLC-Chip. J Sep Sci 30:1427-34.
45. Kłodzińska E, Moravcova D, Jandera P, Buszewski B (2006) Monolithic continuous beds as a new generation of stationary phase for chromatographic and electro-driven separations. J Chromatogr A 1109:51-9.
46. Wu R, Hu L, Wang F, Ye M, Zou H (2007) Recent development of monolithic stationary phases with emphasis on microscale chromatographic separation. J Chromatogr A 1184:369-92.

47. Peterson DS (2005) Solid supports for micro analytical systems. Lab Chip 5:132-9.
48. Choi S, Park JK (2005) Microfluidic system for dielectrophoretic separation based on a trapezoidal electrode array. Lab Chip 5:1161-7.
49. Tai CH, Hsiung SK, Chen CY, Tsai ML, Lee GB (2007) Automatic microfluidic platform for cell separation and nucleus collection. Biomed Microdevices 9:533-43
50. Borgatti M, Altomare L, Baruffa M, Fabbri E, Breveglieri G, Feriotto G, Manaresi N, Medoro G, Romani A, Tartagni M, Gambari R, Guerrieri R (2005) Separation of white blood cells from erythrocytes on a dielectrophoresis (DEP) based 'Lab-on-a-chip' device. Int J Mol Med 15:913-20.
51. Borgatti M, Altomare L, Abonnec M, Fabbri E, Manaresi N, Medoro G, Romani A, Tartagni M, Nastruzzi C, Di Croce S, Tosi A, Mancini I, Guerrieri R, Gambari R (2005) Dielectrophoresis-based 'Lab-on-a-chip' devices for programmable binding of microspheres to target cells. Int J Oncol 27:1559-66.
52. Wang L, Flanagan LA, Monuki E, Jeon NL, Lee AP (2007) Dielectrophoresis switching with vertical sidewall electrodes for microfluidic flow cytometry. Lab Chip 7:1114-20.
53. Yang L., Banada PP, Chatni MR, Seop Lim K, Bhunia AK, Ladisch M, Bashir RA (2006) Multifunctional micro-fluidic system for dielectrophoretic concentration coupled with immuno-capture of low numbers of Listeria monocytogenes. Lab Chip 6:896-905.
54. Wiklund M, Günther C, Lemor R, Jäger M, Fuhr G, Hertz HM (2006) Ultrasonic standing wave manipulation technology integrated into a dielectrophoretic chip. Lab Chip 6:1537-44.
55. Ryu C, Lee K, Yoo C, Seong WK, Oh HB (2003) Sensitive and rapid quantitative detection of anthrax spores isolated from soil samples by qPCR. Microbiol Immunol 47:693-9.
56. Porteous LA, Armstrong JL (1993) A simple mini-method to extract DNA directly from soil for use with polymerase chain reaction amplification. Curr Microbiol 27:115-8.
57. Kabir S, Rajendran N, Amemiya T, Itoh K (2003) Q- quantitative PCR assay on bacterial DNA: In a model soil system and environmental samples. J Gen Appl Microbiol 49:101-9.
58. Hadd, A. and S. Jovanovich. Methods and apparatus for template capture and normalization for submicroliter reaction. December 3, 2002. U. S. Patent 6,489,112.
59. Yoza B, Matsumoto M, Matsunaga T (2002) DNA extraction using modified bacterial magnetic particles in the presence of amino silane compound. J Biotechnol 94:217-24.
60. Cady NC, Stelick S, Batt CA (2003) Nucleic acid purification using microfabricated silicon structures. Biosens Bioelectron 19:59-66.
61. Christel LA, Petersen K, McMillan W, Northrup MA (1999) Rapid, automated nucleic acid probe assays using silicon microstructures for nucleic acid concentration. J Biomech Eng 121:22-7.

62. Liu Y, Cady NC, Batt CA (2007) A plastic microchip for nucleic acid purification. Biomed Microdevices 9:769-76.
63. Nakagawa T, Tanaka T, Niwa D, Osaka T, Takeyama H, Matsunaga T (2005) Fabrication of amino silane-coated microchip for DNA extraction from whole blood. J Biotechnol 116:105-11.
64. Chung YC, Jan MS, Lin YC, Lin JH, Cheng WC, Fan CY (2004) Microfluidic chip for high efficiency DNA extraction. Lab Chip 4:141-7.
65. Chen X, Cui D, Liu C, Li H, Chen J (2007) Continuous flow microfluidic device for cell separation, cell lysis and DNA purification. Anal Chim Acta 584:237-43.
66. Malik A (2002) Advances in sol-gel based columns for capillary electrochromatography: sol-gel open-tubular columns. Electrophoresis 23:3973-92.
67. Svec, F (2006) Less common applications of monoliths: preconcentration and solid-phase extraction. J Chromatogr B 841:52-64.
68. Breadmore MC, Wolfe KA, Arcibal IG, Leung WK, Dickson D, Giordano BC, Power ME, Ferrance JP, Feldman SH, Norris PM, Landers JP (2003) Microchip-based purification of DNA from biological samples. Anal Chem 75:1880-6.
69. Tian H, Hühmer AF, Landers JP (2000) Evaluation of silica resins for direct and efficient extraction of DNA from complex biological matrices in a miniaturized format. Anal Biochem 283:175-91.
70. Wen J, Guillo C, Ferrance JP, Landers JP (2006) DNA extraction using a tetramethyl orthosilicate-grafted photopolymerized monolithic solid phase. Anal Chem 78:1673-81.
71. Wu Q, Bienvenue JM, Hassan BJ, Kwok YC, Giordano BC, Norris PM, Landers JP, Ferrance JP (2006) Microchip-based macroporous silica sol-gel monolith for efficient isolation of DNA from clinical samples. Anal Chem 78:5704-10.
72. Wen J, Guillo C, Ferrance JP, Landers JP (2007) Microfluidic-based DNA purification in a two-stage, dual-phase microchip containing a reversed-phase and a photopolymerized monolith. Anal Chem 79:6135-42.
73. Wen J, Guillo C, Ferrance JP, Landers JP (2007) Microfluidic chip-based protein capture from human whole blood using octadecyl (C18) silica beads for nucleic acid analysis from large volume samples. J Chromatogr A 1171:29-36.
74. Grodzinski P, Liu R, Yang J, Ward MD (2004) Microfluidic system integration in sample preparation chip-sets - a summary. In: Conf Proc IEEE Eng Med Biol Soc 4:2615-8.
75. Peoples MC, Karnes HT (in press) Microfluidic immunoaffinity separations for bioanalysis. J Chromatogr B.
76. Stevens KA, Jaykus LA (2004) Bacterial separation and concentration from complex sample matrices: a review. Crit Rev Microbiol 30:7-24.
77. O'Brien SB, Duffy G, Daly D, Sheridan JJ, Blair IS, McDowell DA (2005) Detection and recovery rates achieved using direct plate and enrichment/immunomagnetic separation methods for Escherichia coli O157:H7 in minced beef and on bovine hide. Lett Appl Microbiol 41:88-93.

78. Fu Z, Rogelj S, Kieft TL (2005) Rapid detection of Escherichia coli O157:H7 by immunomagnetic separation and qPCR. Int J Food Microbiol 99:47-57.
79. Pyle BH, Broadaway SC, McFeters GA (1999) Sensitive detection of Escherichia coli O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. Appl Environ Microbiol 65:1966-72.
80. Varshney M, Yang L, Su XL, Li Y (2005) Magnetic nanoparticle-antibody conjugates for the separation of Escherichia coli O157:H7 in ground beef. J Food Prot 68:1804-11.
81. Rajkovic A, El-Moualij B, Uyttendaele M, Brolet P, Zorzi W, Heinen E, Foubert E, Debevere J (2006) Immunoquantitative qPCR for detection and quantification of Staphylococcus aureus enterotoxin B in foods. Appl Environ Microbiol 72:6593-9.
82. Ramadan Q, Samper V, Poenar D, Yu C (2006) Magnetic-based microfluidic platform for biomolecular separation. Biomed Microdevices 8:151-8.
83. Xia N, Hunt TP, Mayers BT, Alsberg E, Whitesides GM, Westervelt RM, Ingber DE (2006) Combined microfluidic-micromagnetic separation of living cells in continuous flow. Biomed Microdevices 8:299-308.
84. Furdui VI, Harrison DJ (2004) Immunomagnetic T cell capture from blood for PCR analysis using microfluidic systems. Lab Chip 4:614-8.
85. Peruski AH, Peruski LF Jr (2003) Immunological methods for detection and identification of infectious disease and biological warfare agents. Clin Diagn Lab Immunol 10:506-13.
86. Andreotti PE, Ludwig GV, Peruski AH, Tuite JJ, Morse SS, Peruski LF Jr (2003) Immunoassay of infectious agents. Biotechniques 35:850-9.
87. Fischer NO, Tarasow TM, Tok JB (2007) Heightened sense for sensing: recent advances in pathogen immunoassay sensing platforms. Analyst 132:187-91.
88. Bange A, Halsall HB, Heineman WR (2005) Microfluidic immunosensor systems. Biosens Bioelectron 20:2488-503.
89. Kim MS, Doyle MP (1992) Dipstick immunoassay to detect enterohemorrhagic Escherichia coli O157:H7 in retail ground beef. Appl Environ Microbiol 58:1764-7.
90. Grunow R, Splettstoesser W, McDonald S, Otterbein C, O'Brien T, Morgan C, Aldrich J, Hofer E, Finke EJ, Meyer H (2000) Detection of Francisella tularensis in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. Clin Diagn Lab Immunol 7:86-90.
91. Herr AE, Hatch AV, Throckmorton DJ, Tran HM, Brennan JS, Giannobile WV, Singh AK (2007) Microfluidic immunoassays as rapid saliva-based clinical diagnostics. Proc Natl Acad Sci U S A 104:5268-73.
92. Schotte U, Langfeldt N, Peruski AH, Meyer H (2002) Detection of staphylococcal enterotoxin B (SEB) by enzyme-linked immunosorbent assay and by a rapid hand-held assay. Clin Lab 48:395-400.
93. Moorthy J, Mensing GA, Kim D, Mohanty S, Eddington DT, Tepp WH, Johnson EA, Beebe DJ (2004) Microfluidic tectonics platform: A colorimetric, disposable botulinum toxin enzyme-linked immunosorbent assay system. Electrophoresis 25:1705-13.

94. Padhye NV, Doyle MP (1991) Rapid procedure for detecting enterohemorrhagic Escherichia coli O157:H7 in food. Appl Environ Microbiol 57:2693-8.
95. Medina MB (2006) Development of a fluorescent latex microparticle immunoassay for the detection of staphylococcal enterotoxin B (SEB). J Agric Food Chem 54:4937-42.
96. Khan AS, Cao CJ, Thompson RG, Valdes JJ (2003) A simple and rapid fluorescence-based immunoassay for the detection of staphylococcal enterotoxin B. Mol Cell Probes 17:125-6.
97. Alefantis T, Grewal P, Ashton J, Khan AS, Valdes JJ, Del Vecchio VG (2004) A rapid and sensitive magnetic bead-based immunoassay for the detection of staphylococcal enterotoxin B for high-through put screening. Mol Cell Probes 18:379-82.
98. Mangru S, Bentz BL, Davis TJ, Desai N, Stabile PJ, Schmidt JJ, Millard CB, Bavari S, Kodukula K (2005) Integrated bioassays in microfluidic devices: botulinum toxin assays. J Biomol Screen 10:788-94.
99. Lim CT, Zhang Y (2007) Bead-based microfluidic immunoassays: the next generation. Biosens Bioelectron 22:1197-204.
100. Gao Y, Hu G, Lin FY, Sherman PM, Li D (2005) An electrokinetically-controlled immunoassay for simultaneous detection of multiple microbial antigens. Biomed Microdevices 7:301-12.
101. Bai Y, Koh CG, Boreman M, Juang YJ, Tang IC, Lee LJ, Yang ST (2006) Surface modification for enhancing antibody binding on polymer-based microfluidic device for enzyme-linked immunosorbent assay. Langmuir 22:9458-67.
102. Czajka J, Batt CA (1996) A solid phase fluorescent capillary immunoassay for the detection of Escherichia coli O157:H7 in ground beef and apple cider. J Appl Bacteriol 81:601-7.
103. Peoples MC, Phillips TM, Karnes HT (2007) A capillary-based microfluidic instrument suitable for immunoaffinity chromatography. J Chromatogr B 848:200-7.
104. Herrmann M, Roy E, Veres T, Tabrizian M (2007) Microfluidic ELISA on non-passivated PDMS chip using magnetic bead transfer inside dual networks of channels. Lab Chip 7:1546-52.
105. Herrmann M, Veres T, Tabrizian M (2006) Enzymatically-generated fluorescent detection in micro-channels with internal magnetic mixing for the development of parallel microfluidic ELISA. Lab Chip 6:555-60.
106. Liu RH (2004) Integrated microfluidic biochips for immunoassay and DNA bioassays. In: Conf Proc IEEE Eng Med Biol Soc 7:5394.
107. Taitt CR, Golden JP, Shubin YS, Shriver-Lake LC, Sapsford KE, Rasooly A, Ligler FS (2004) A portable array biosensor for detecting multiple analytes in complex samples. Microb Eco 47:175-85.
108. Ligler FS, Taitt CR, Shriver-Lake LC, Sapsford KE, Shubin Y, Golden JP (2003) Array biosensor for detection of toxins. Anal Bioanal Chem 377:469-77.

109. Taitt CR, Shubin YS, Angel R, Ligler FS (2004) Detection of Salmonella enterica serovar typhimurium by using a rapid, array-based immunosensor: Appl Environ Microbiol 70:152-8.
110. McBride MT, Gammon S, Pitesky M, O'Brien TW, Smith T, Aldrich J, Langlois RG, Colston B, Venkateswaran KS (2003) Multiplexed liquid arrays for simultaneous detection of simulants of biological warfare agents. Anal Chem 75:1924-30.
111. Yang JM, Bell J, Huang Y, Tirado M, Thomas D, Forster AH, Haigis RW, Swanson, PD Wallace RB, Martinsons B, Krihak M (2002) An integrated, stacked microlaboratory for biological agent detection with DNA and immunoassays. Biosensors & Bioelectronics 17:605-618.
112. Gatto-Menking DL, Yu H, Bruno JG, Goode MT, Miller M, Zulich AW (1995) Sensitive detection of biotoxoids and bacterial spores using an immunomagnetic electrochemiluminescence sensor. Biosens Bioelectron 10:501-7.
113. Yu H, Raymonda JW, McMahon TM, Campagnari AA (2000) Detection of biological threat agents by immunomagnetic microsphere-based solid phase fluorogenic- and electro-chemiluminescence. Biosens Bioelectron 14:829-40.
114. Kijek TM, Rossi CA, Moss D, Parker RW, Henchal EA (2000) Rapid and sensitive immunomagnetic-electrochemiluminescent detection of staphyloccocal enterotoxin B. J Immunol Methods 236:9-17.
115. Rivera VR, Gamez FJ, Keener WK, White JA, Poli MA (2006) Rapid detection of Clostridium botulinum toxins A, B, E, and F in clinical samples, selected food matrices, and buffer using paramagnetic bead-based electrochemiluminescence detection. Anal Biochem 353:248-56.
116. Keener WK, Rivera VR, Young CC, Poli MA (2006) An activity-dependent assay for ricin and related RNA N-glycosidases based on electrochemiluminescence. Anal Biochem 357:200-7.
117. Smith DR, Rossi CA, Kijek TM, Henchal EA, Ludwig GV (2001) Comparison of dissociation-enhanced lanthanide fluorescent immunoassays to enzyme-linked immunosorbent assays for detection of staphylococcal enterotoxin B, Yersinia pestis-specific F1 antigen, and Venezuelan equine encephalitis virus. Clin Diagn Lab Immunol 8:1070-5.
118. Peruski AH, Johnson LH 3rd, Peruski LF Jr (2002) Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays. J Immunol Methods 263:35-41.
119. Baselt DR, Lee GU, Natesan M, Metzger SW, Sheehan PE, Colton RJ (1998) A biosensor based on magnetoresistance technology. Biosens Bioelectron 13:731-9.
120. Millen RL, Kawaguchi T, Granger MC, Porter MD, Tondra M (2005) Giant magnetoresistive sensors and superparamagnetic nanoparticles: a chip-scale detection strategy for immunosorbent assays. Anal Chem 77:6581-7.
121. Rowe CA, Tender LM, Feldstein MJ, Golden JP, Scruggs SB, MacCraith BD, Cras JJ, Ligler FS.(1999) Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. Anal Chem 71:3846-52.
122. Mulvaney SP,Cole CL, Kniller MD, Malito M, Tamanaha CR, Rife JC, Stanton MW, Whitman LJ (2007) Rapid, femtomolar bioassays in complex matri-

ces combining microfluidics and magnetoelectronics. Biosens Bioelectron 23:191-200.
123. Deng Y, Schwegler EE, Gravenstein S (2005) Proteomics for biodefense applications: progress and opportunities. Expert Rev Proteomics 2:203-13.
124. Chen X, Wu H, Mao C, Whitesides GM (2002) A prototype two-dimensional capillary electrophoresis system fabricated in poly(dimethylsiloxane). Anal Chem 74:1772-8.
125. Zhang C, Crasta O, Cammer S, Will R, Kenyon R, Sullivan D, Yu Q, Sun W, Jha R, Liu D, Xue T, Zhang Y, Moore M, McGarvey P, Huang H, Chen Y, Zhang J, Mazumder R, Wu C, Sobral B (2008) An emerging cyberinfrastructure for biodefense pathogen and pathogen host data. Nucleic Acids Res 36:D884-91.
126. http://www.proteomicsresource.org/
127. Lion N, Rohner TC, Dayon L, Arnaud IL, Damoc E, Youhnovski N, Wu ZY, Roussel C, Josserand J, Jensen H, Rossier JS, Przybylski M, Girault HH. (2003) Microfluidic systems in proteomics. Electrophoresis 24:3533-62.
128. Lazar IM, Grym J, Foret F (2006) Microfabricated devices: A new sample introduction approach to mass spectrometry. Mass Spectrom Rev 25:573-94.
129. Stachowiak JC, Shugard EE, Mosier BP, Renzi RF, Caton PF, Ferko SM, Van de Vreugde JL, Yee DD, Haroldsen BL, VanderNoot VA (2007) Autonomous microfluidic sample preparation system for protein profile-based detection of aerosolized bacterial cells and spores. Anal Chem 79:5763-70.
130. Mattoon D, Michaud G, Merkel J, Schweitzer B (2005) Biomarker discovery using protein microarray technology platforms: antibody-antigen complex profiling. Expert Rev Proteomics 2:879-89.
131. Huelseweh B, Ehricht R, Marschall HJ (2006) A simple and rapid protein array based method for the simultaneous detection of biowarfare agents. Proteomics 6:2972-81.
132. Petrenko VA, Vodyanoy VJ (2003) Phage display for detection of biological threat agents. J Microbiol Methods 53:253-62.
133. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia. Science 230:1350–1354.
134. Ratcliff RM, Chang G, Kok T, Sloots TP (2007) Molecular diagnosis of medical viruses. Curr Issues Mol Biol 9:87-102.
135. Mothershed EA, Whitney AM (2006) Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. Clin Chim Acta 363:206-20.
136. Barken KB, Haagensen JA, Tolker-Nielsen T (2007) Advances in nucleic acid-based diagnostics of bacterial infections. Clin Chim Acta 384:1-11.
137. Ivnitski D, O'Neil DJ, Gattuso A, Schlicht R, Calidonna M, Fisher R (2003) Nucleic acid approaches for detection and identification of biological warfare and infectious disease agents. Biotechniques 35:862-9.
138. Mackay IM (2004) qPCR in the microbiology laboratory. Clin Microbiol Infect 10:190-212.

139. Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR 3rd, Smith TF (2006) qPCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 19:165-256.
140. McDonald R, Cao T, Borschel R (2001) Multiplexing for the detection of multiple biowarfare agents shows promise in the field. Mil Med 166:237-9.
141. Ramisse V, Patra G, Garrigue H, Guesdon JL, Mock M (1996) Identification and characterization of Bacillus anthracis by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA. FEMS Microbiol Lett 145:9-16.
142. Lee MA, Brightwell G, Leslie D, Bird H, Hamilton A (1999) Fluorescent detection techniques for Q- multiplex strand specific detection of Bacillus anthracis using rapid PCR. J Appl Microbiol 87:218-23.
143. Bell CA, Uhl JR, Hadfield TL, David JC, Meyer RF, Smith TF, Cockerill FR 3rd (2002) Detection of Bacillus anthracis DNA by LightCycler PCR. J Clin Microbiol 40:2897-902.
144. Uhl JR, Bell CA, Sloan LM, Espy MJ, Smith TF, Rosenblatt JE, Cockerill FR 3rd (2002) Application of rapid-cycle Q- polymerase chain reaction for the detection of microbial pathogens: the Mayo-Roche Rapid Anthrax Test. Mayo Clin Proc 77:673-80.
145. Ryu C, Lee K, Yoo C, Seong WK, Oh HB (2003) Sensitive and rapid quantitative detection of anthrax spores isolated from soil samples by qPCR. Microbiol Immunol 47:693-9.
146. Makino S, Cheun HI (2003) Application of the qPCR for the detection of airborne microbial pathogens in reference to the anthrax spores. J Microbiol Methods 53:141-7.
147. McKillip JL, Drake M (2004) Real-time nucleic acid-based detection methods for pathogenic bacteria in food. J Food Prot 67:823-32.
148. Varma-Basil M, El-Hajj H, Marras SA, Hazbón MH, Mann JM, Connell ND, Kramer FR, Alland D (2004) Molecular beacons for multiplex detection of four bacterial bioterrorism agents. Clin Chem 50:1060-2.
149. Skottman T, Piiparinen H, Hyytiäinen H, Myllys V, Skurnik M, Nikkari S (2007) Simultaneous qPCR detection of Bacillus anthracis, Francisella tularensis and Yersinia pestis. Eur J Clin Microbiol Infect Dis 26:207-11.
150. Selvapandiyan A, Stabler K, Ansari NA, Kerby S, Riemenschneider J, Salotra P, Duncan R, Nakhasi HL (2005) A novel semiquantitative fluorescence-based multiplex polymerase chain reaction assay for rapid simultaneous detection of bacterial and parasitic pathogens from blood. J Mol Diagn 7:268-75.
151. Kim K, Seo J, Wheeler K, Park C, Kim D, Park S, Kim W, Chung SI, Leighton T (2005) Rapid genotypic detection of Bacillus anthracis and the Bacillus cereus group by multiplex qPCR melting curve analysis. FEMS Immunol Med Microbiol 43:301-10.
152. Christensen DR, Hartman LJ, Loveless BM, Frye MS, Shipley MA, Bridge DL, Richards MJ, Kaplan RS, Garrison J, Baldwin CD, Kulesh DA, Norwood DA (2006) Detection of biological threat agents by qPCR: comparison of as-

say performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler platforms. Clin Chem 52:141-5.
153. Ulrich MP, Christensen DR, Coyne SR, Craw PD, Henchal EA, Sakai SH, Swenson D, Tholath J, Tsai J, Weir AF, Norwood DA (2006) Evaluation of the Cepheid GeneXpert system for detecting Bacillus anthracis. J Appl Microbiol 100:1011-6.
154. Zhang C, Xing D (2007) Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. Nucleic Acids Res 35:4223-37.
155. Kricka LJ, Wilding P (2003) Microchip PCR. Anal Bioanal Chem 377:820-5.
156. Wittwer CT, Fillmore GC, Hillyard DR (1989) Automated polymerase chain reaction in capillary tubes with hot air. Nucleic Acids Res 17: 4353-4357.
157. Wittwer CT, Fillmore GC, Garling DJ (1990) Minimizing the time required for DNA amplification by efficient heat transfer to small samples. Anal Biochem 186:328-31.
158. Friedman NA, Meldrum D (1998) Capillary Tube Resistive Thermal Cycling. Anal Chem 70:2997-3002.
159. Meldrum DR, Evensen HT, Pence WH, Moody SE, Cunningham DL, Wiktor PJ (2000) ACAPELLA-1K, a capillary-based submicroliter automated fluid handling system for genome analysis. Genome Res 10:95-104.
160. Northrup MA, Ching MT, White RM, Watson RT (1993) DNA amplification with a microfabricated reaction chamber. In: Digest of technical papers: transducers 1993 (Proc. 7th mt. conf. on solidstate sensors and actuators). New York Institute of Electrical and Electronic Engineers, 1993:924-6.
161. Wilding P, Shoffner MA, Kricka LJ (1994) PCR in a silicon microstructure. Clin Chem 40:1815-8.
162. Nagai H, Murakami Y, Morita Y, Yokoyama K, Tamiya E (2001) Development of a microchamber array for picoliter PCR. Anal Chem 73:1043-7.
163. Woolley AT, Hadley D, Landre P, deMello AJ, Mathies RA, Northrup MA (1998) Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device. J Forensic Sci 43:315-9.
164. Belgrader P, Benett W, Hadley D, Richards J, Stratton P, Mariella R Jr, Milanovich F (1999) PCR detection of bacteria in seven minutes. Science 284:449-50.
165. Northrup MA, Benett B, Hadley D, Landre P, Lehew S, Richards J, Stratton P (1998) A miniature analytical instrument for nucleic acids based on micromachined silicon reaction chambers. Anal Chem 70:918-22.
166. Belgrader P, Young S, Yuan B, Primeau M, Christel LA, Pourahmadi F, Northrup MA (2000) A battery-powered notebook thermal cycler for rapid multiplex real-time PCR analysis. Biosens Bioelectron 14:849-52.
167. Neuzil P, Pipper J, Hsieh TM (2006) Disposable Q- microPCR device: lab-on-a-chip at a low cost. Mol Biosyst 2:292-8.
168. Neuzil P, Zhang C, Pipper J, Oh S, Zhuo L (2006) Ultra fast miniaturized qPCR: 40 cycles in less than six minutes. Nucleic Acids Res 34:e77.

169. Wang Z, Sekulovic A, Kutter JP, Bang DD, Wolff A (2006) Towards a portable microchip system with integrated thermal control and polymer waveguides for qPCR. Electrophoresis 27:5051-8.
170. Oh KW, Park C, Namkoong K, Kim J, Ock KS, Kim S, Kim YA, Cho YK, Ko C (2005) World-to-chip microfluidic interface with built-in valves for multichamber chip-based PCR assays. Lab Chip 5:845-50.
171. Koh CG, Tan W, Zhao MQ, Ricco AJ, Fan ZH (2003) Integrating polymerase chain reaction, valving, and electrophoresis in a plastic device for bacterial detection. Anal Chem 75:4591-8.
172. Gong H, Ramalingam N, Chen L, Che J, Wang Q, Wang Y, Yang X, Yap PH, Neo CH (2006) Microfluidic handling of PCR solution and DNA amplification on a reaction chamber array biochip. Biomed Microdevices 8:167-76.
173. Liao CS, Lee GB, Wu JJ, Chang CC, Hsieh TM, Huang FC, Luo CH.(2005) Micromachined polymerase chain reaction system for multiple DNA amplification of upper respiratory tract infectious diseases. Biosens Bioelectron 20:1341-8.
174. Oda RP, Strausbauch MA, Huhmer AF, Borson N, Jurrens SR, Craighead J, Wettstein PJ, Eckloff B, Kline B, Landers JP (1998) Infrared-mediated thermocycling for ultrafast polymerase chain reaction amplification of DNA. Anal Chem 70:4361-8.
175. Roper MG, Easley CJ, Legendre LA, Humphrey JA, Landers JP.(2007) Infrared temperature control system for a completely noncontact polymerase chain reaction in microfluidic chips. Anal Chem 79:1294-300.
176. Legendre LA, Bienvenue JM, Roper MG, Ferrance JP, Landers JP (2006) A simple, valveless microfluidic sample preparation device for extraction and amplification of DNA from nanoliter-volume samples. Anal Chem 78:1444-51.
177. Hashimoto M, Barany F, Soper SA (2006) Polymerase chain reaction/ligase detection reaction/hybridization assays using flow-through microfluidic devices for the detection of low-abundant DNA point mutations. Biosens Bioelectron 21:1915-23.
178. Obeid PJ, Christopoulos TK, Crabtree HJ, Backhouse CJ (2003) Microfabricated device for DNA and RNA amplification by continuous-flow polymerase chain reaction and reverse transcription-polymerase chain reaction with cycle number selection. Anal Chem 75:288-95.
179. Münchow G, Dadic D, Doffing F, Hardt S, Drese KS (2005) Automated chip-based device for simple and fast nucleic acid amplification. Expert Rev Mol Diagn 5:613-20.
180. Nagai H, Murakami Y, Yokoyama K, Tamiya E.(2001) High-throughput PCR in silicon based microchamber array. Biosens Bioelectron 16:1015-9.
181. Mitterer G, Schmidt WM (2006) Microarray-based detection of bacteria by on-chip PCR. Methods Mol Biol 345:37-51.
182. Mitterer G, Huber M, Leidinger E, Kirisits C, Lubitz W, Mueller MW, Schmidt WM (2004) Microarray-based identification of bacteria in clinical samples by solid-phase PCR amplification of 23S ribosomal DNA sequences. J Clin Microbiol 42:1048-57.

183. Belgrader P, Benett W, Hadley D, Long G, Mariella R Jr, Milanovich F, Nasarabadi S, Nelson W, Richards J, Stratton P (1998) Rapid pathogen detection using a microchip PCR array instrument. Clin Chem 44:2191-4.
184. Northrup MA, Benett B, Hadley D, Landre P, Lehew S, Richards J, Stratton P (1998) A miniature analytical instrument for nucleic acids based on micromachined silicon reaction chambers. Anal Chem 70:918-922.
185. Higgins JA, Nasarabadi S, Karns JS, Shelton DR, Cooper M, Gbakima A, Koopman RP (2003) A handheld real time thermal cycler for bacterial pathogen detection. Biosens Bioelectron 18:1115-23.
186. McBride MT, Masquelier D, Hindson BJ, Makarewicz AJ, Brown S, Burris K, Metz T, Langlois RG, Tsang KW, Bryan R, Anderson DA, Venkateswaran KS, Milanovich FP, Colston BW Jr (2003) Autonomous detection of aerosolized Bacillus anthracis and Yersinia pestis. Anal Chem 75:5293-9.
187. Arroyo E Wheeler, EK Shediac R, Hindson B, Nasarabadi S, Vrankovich G, Bell P, Bailey C, Sheppod T, Christian AT (2005) Flow through PCR module of BioBriefcase. Smart Medical and Biomedical Sensor Technology III. Edited by Cullum BM, Chance CJ. In: Proceedings of the SPIE, Volume 6007, pp. 243-251.
188. Wilson WJ, Erler AM, Nasarabadi SL, Skowronski EW, Imbro PM. (2005) A multiplexed PCR-coupled liquid bead array for the simultaneous detection of four biothreat agents. Mol Cell Probes 19:137-44.
189. McAvin JC, McConathy MA, Rohrer AJ, Huff WB, Barnes WJ, Lohman KL (2003) A Q- fluorescence polymerase chain reaction assay for the identification of Yersinia pestis using a field-deployable thermocycler. Mil Med 168:852-5.
190. Beer NR, Hindson BJ, Wheeler EK, Hall SB, Rose KA, Kennedy IM, Colston BW (2007) On-chip, Q-, single-copy polymerase chain reaction in picoliter droplets. Anal Chem 79:8471-5.
191. Liao CS, Lee GB, Liu HS, Hsieh TM, Luo CH (2005) Miniature QPCR system for diagnosis of RNA-based viruses. Nucleic Acids Res 33:e156.
192. Marcus JS, Anderson WF, Quake SR (2006) Parallel picoliter qPCR assays using microfluidics. Anal Chem 78:956-8.
193. Deiman B, van Aarle P, Sillekens P (2002) Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol 20:163-79.
194. Spargo CA, Fraiser MS, Van Cleve M, Wright DJ, Nycz CM, Spears PA, Walker GT (1996) Detection of M. tuberculosis DNA using thermophilic strand displacement amplification. Mol Cell Probes 10:247-56.
195. Hellyer TJ, Nadeau JG (2004) Strand displacement amplification: a versatile tool for molecular diagnostics. Expert Rev Mol Diagn 4:251-61.
196. Nadeau JG, Pitner JB, Linn CP, Schram JL, Dean CH, Nycz CM (1999) Anal Biochem 276:177-87.
197. Wang SS, Thornton K, Kuhn AM, Nadeau JG, Hellyer TJ (2003) Homogeneous Q- detection of single-nucleotide polymorphisms by strand displacement amplification on the BD ProbeTec ET system. Clin Chem 49:1599-607.

198. Van Ness J, Van Ness LK, Galas DJ (2003) Isothermal reactions for the amplification of oligonucleotides. Proc Natl Acad Sci U S A 100:4504-9.
199. Tan E, Wong J, Nguyen D, Zhang Y, Erwin B, Van Ness LK, Baker SM, Galas DJ, Niemz A (2005) Isothermal DNA amplification coupled with DNA nanosphere-based colorimetric detection. Anal Chem 77:7984-92
200. Notomi T,Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hese T (2000) Loop-mediated isothermal amplification of DNA. Nuc Acids Res 28:e63-i-vii.
201. Poon LB, Wong B, Ma, Chan K, Chow L, Abeyewickreme W, Tanngpukdee N, Yuen K, Guan Y, Looareesuwan S, Peiris J (2006) Sensitive and inexpensive molecular test for Falciparum malaria: Detecting Plasmodium falciparum DNA directly from heat-treated blood by loop-mediated isothermal amplification. Clin. Chem 52: 303-306.
202. Enosawa MS, Kageyama S, Sawai K, Watanabe K, Notomi T, Onoe S, Mori Y, Yokomizo Y (2003) Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured Mycobacterium avium subsp. Paratuberculosis. J Clin Microbiol 41:4359-4365.
203. Dean FB, Nelson JR, Giesler TL, Lasken RS (2001) Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res 11:1095-9.
204. Nelson JR, Cai YC, Giesler TL, Farchaus JW, Sundaram ST, Ortiz-Rivera M, Hosta LP, Hewitt PL, Mamone JA, Palaniappan C, Fuller CW (2002) TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. Biotechniques Jun;Suppl:44-7.
205. Reagin MJ, Giesler TL, Merla AL, Resetar-Gerke JM, Kapolka KM, Mamone JA (2003) TempliPhi: A sequencing template preparation procedure that eliminates overnight cultures and DNA purification. J Biomol Tech 14:143-8.
206. Hutchison CA 3rd, Smith HO, Pfannkoch C, Venter JC (2005) Cell-free cloning using phi29 DNA polymerase. Proc Natl Acad Sci U S A 102:17332-6.
207. Hashsham SA, Wick LM, Rouillard JM, Gulari E, Tiedje JM (2004) Potential of DNA microarrays for developing parallel detection tools (PDTs) for microorganisms relevant to biodefense and related research needs. Biosens Bioelectron 20:668-83.
208. Stenger DA, Andreadis JD, Vora GJ, Pancrazio JJ (2002) Potential applications of DNA microarrays in biodefense-related diagnostics. Curr Opin Biotechnol 13:208-12.
209. Volokhov D, Pomerantsev A, Kivovich V, Rasooly A, Chizhikov V (2004) Identification of Bacillus anthracis by multiprobe microarray hybridization. Diagn Microbiol Infect Dis 49:163-71.
210. Sergeev N, Distler M, Courtney S, Al-Khaldi SF, Volokhov D, Chizhikov V, Rasooly A (2004) Multipathogen oligonucleotide microarray for environmental and biodefense applications. Biosens Bioelectron 20:684-98.
211. Situma C, Hashimoto M, Soper SA (2006) Merging microfluidics with microarray-based bioassays. Biomol Eng 23:213-31.
212. Anderson RC, Su X, Bogdan GJ, Fenton J (2000) A miniature integrated device for automated multistep genetic assays. Nucleic Acids Res 28:E60.

213. Soper SA, Hashimoto M, Situma C, Murphy MC, McCarley RL, Cheng YW, Barany F (2005) Fabrication of DNA microarrays onto polymer substrates using UV modification protocols with integration into microfluidic platforms for the sensing of low-abundant DNA point mutations. Methods 37:103-13.
214. Liu RH, Yang J, Lenigk R, Bonanno J, Grodzinski P (2004) Self-contained, fully integrated biochip for sample preparation, polymerase chain reaction amplification, and DNA microarray detection. Anal Chem 76:1824-31.
215. Liu RH, Lodes MJ, Nguyen T, Siuda T, Slota M, Fuji HS, McShea A (2006) Validation of a fully integrated microfluidic array device for influenza A subtype identification and sequencing. Anal Chem 78:4184-93.
216. Liu RH, Nguyen T, Schwarzkopf K, Fuji HS, Petrova A, Siuda T, Peyvan K, Bizak M, Danley D, McShea A (2006) Fully integrated miniature device for automated gene expression DNA microarray processing. Anal Chem 78:1980-6.
217. Keen-Kim D, Grody WW, Richards CS (2006) Microelectronic array system for molecular diagnostic genotyping: Nanogen NanoChip 400 and molecular biology workstation. Expert Rev Mol Diagn 6:287-94.
218. Sosnowski R, Heller MJ, Tu E, Forster AH, Radtkey R (2002) Active microelectronic array system for DNA hybridization, genotyping and pharmacogenomic applications. Psychiatr Genet 12:181-92.
219. Borowsky J, Collins GE (2007) Chemical and biological threat-agent detection using electrophoresis-based lab-on-a-chip devices. Analyst 132:958-62.
220. Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E et al (1987) Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235:1616-22.
221. Adair DM, Worsham PL, Hill KK, Klevytska AM, Jackson PJ, Friedlander AM, Keim PJ (2000) Diversity in a variable-number tandem repeat from Yersinia pestis. Clin Microbiol 38:1516-9.
222. Skuce RA, McCorry TP, McCarroll JF, Roring SM, Scott AN, Brittain D, Hughes SL, Hewinson RG, Neill SD (2002) Discrimination of M. tuberculosis complex bacteria using novel VNTR-PCR targets. Microbiology 148:519-2.
223. Keim P, LB Price AM, Klevytska, KL Smith, JM Schupp, R Okinaka, PJ Jackson, Hugh- Jones ME (2000) Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within Bacillus anthracis. J Bacteriol 182:2928-36.
224. Hoffmaster AR, Fitzgerald CC, Ribot E, Mayer LW, Popovic T (2002) Molecular subtyping of Bacillus anthracis and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg. Infect Dis 8:1111-6.
225. Valjevac S, Hilaire V, Lisanti O, Ramisse F, Hernandez E, Cavallo JD, Pourcel C, Vergnaud G (2005) Comparison of minisatellite polymorphisms in the Bacillus cereus complex: a simple assay for large-scale screening and identification of strains most closely related to Bacillus anthracis. Appl Environ Microbiol 71:6613-23
226. Stratilo CW, Lewis CT, Bryden L, Mulvey MR, Bader D (2006) Single-nucleotide repeat analysis for subtyping Bacillus anthracis isolates. J Clin Microbiol 44:777-82.

227. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, et al (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407-14.
228. Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M, Kersters K (1996) Evaluation of the DNA fingerprinting method AFLP as an new tool in bacterial taxonomy. Microbiology 142:1881-93.
229. Jackson PJ, Hill KK, Laker MT, Ticknor LO, Keim P (1999) Genetic comparison of Bacillus anthracis and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis. J Appl Microbiol 87:263-9.
230. Lazzaro BP, Sceurman BK, Carney SL, Clark AG (2002) fRFLP and fAFLP: medium-throughput genotyping by fluorescently post-labeling restriction digestion. Biotechniques 33:539-40.
231. Bachem CW, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, ZabeauM, Visser RG (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J 9:745-53.
232. Daffonchio D, Raddadi N, Merabishvili M, Cherif A, Carmagnola L, Brusetti L, Rizzi A, Chanishvili N, Visca P, Sharp R, Borin S (2006) Strategy for identification of Bacillus cereus and Bacillus thuringiensis strains closely related to Bacillus anthracis. Appl Environ Microbiol 72:1295-301.
233. Sacchi CT, Whitney AM, Mayer LW, Morey R, Steigerwalt A, Boras A, Weyant RS, Popovic T (2002) Sequencing of 16S rRNA gene: a rapid tool for identification of Bacillus anthracis. Emerg Infect Dis 8:1117-23.
234. Bavykin SG, Lysov YP, Zakhariev V, Kelly JJ, Jackman J, Stahl DA, Cherni A (2004) Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of Bacillus cereus group microorganisms. J Clin Microbiol 42:3711-30.
235. Ruppitsch W, Stöger A, Indra A, Grif K, Schabereiter-Gurtner C, Hirschl A, Allerberger F (2007) Suitability of partial 16S ribosomal RNA gene sequence analysis for the identification of dangerous bacterial pathogens. J Appl Microbiol 102:852-9.
236. Dolník V. Liu S, Jovanovich S (2000) Capillary electrophoresis on microchips (Review). Electrophoresis 21:41-54.
237. Ugaz VM, Elms RD, Lo RC, Shaikh FA, Burns MA (2004) Microfabricated electrophoresis systems for DNA sequencing and genotyping applications: current technology and future directions. Philos Transact A Math Phys Eng Sci 362:1105-29.
238. Sinville R, Soper SA (2007) High resolution DNA separations using microchip electrophoresis. J Sep Sci 30:1714-28.
239. Chen YH, Wang WC, Young KC, Chang TT, Chen SH (1999) Plastic microchip electrophoresis for analysis of PCR products of hepatitis C virus. Clin Chem 45:1938-43.
240. Harrison DJ, Fluri K, Seiler K, Fan Z, Effenhauser CS, Manz A (1993) Micromachining a Miniaturized Capillary Electrophoresis-Based Chemical Analysis System on a Chip. Science 261:895-897.

241. Woolley AT, Mathies RA (1994) Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. Proc Natl Acad Sci USA 91:11348-11352.
242. Jacobson SC, Hergenroder R, Koutny LB, Ramsey JM (1994) High-speed separations on a microchip. Anal Chem 66:1114-1118.
243. Woolley AT, Sensabaugh GF, Mathies RA (1997) High-Speed DNA Genotyping Using Microfabricated Capillary Array Electrophoresis Chips. Anal Chem 69:2181-2186.
244. Effenhauser CS, Paulus A, Manz A, Widmer HM (1994) High-Speed Separation of Antisense Oligonucleotides on a Micromachined Capillary Electrophoresis Device. Anal. Chem. 66:2949-2953.
245. Liu SR, Shi YN, Ja WW, Mathies RA (1999) Optimization of high-speed DNA sequencing on microfabricated capillary electrophoresis channels. Anal. Chem 71:566-573.
246. Shen Z, Liu X, Long Z, Liu D, Ye N, Qin J, Dai Z, Lin B (2006) Parallel analysis of biomolecules on a microfabricated capillary array chip. Electrophoresis 27:1084-92.
247. Liu S, Ren H, Gao Q, Roach DJ, Loder Jr RT, Armstrong TM, Mao Q, Blaga I, Barker DL, Jovanovich SB (2000) Automated parallel DNA sequencing on multiple channel chips. Proc Natl Acad Sci USA 97: 5369-5374.
248. Kartalov EP, Quake SR (2004) Microfluidic device reads up to four consecutive base pairs in DNA sequencing-by-synthesis. Nucleic Acids Res 32:2873-9.
249. Russom A, Tooke N, Andersson H, Stemme G (2005) Pyrosequencing in a microfluidic flow-through device. Anal Chem 77:7505-11.
250. Seiler K, Harrison D, Manz A (1993) Micromachining a Miniaturized Capillary Electrophoresis-Based Chemical Analysis System on a Chip. Science 261:895-897.
251. Vreeland WN, Williams SJ, Barron AE, Sassi AP (2003) Tandem isotachophoresis-zone electrophoresis via base-mediated destacking for increased detection sensitivity in microfluidic systems. Anal Chem 75:3059-65.
252. Wainright A, Nguyen UT, Bjornson T, Boone TD (2003) Preconcentration and separation of double-stranded DNA fragments by electrophoresis in plastic microfluidic devices. Electrophoresis 24:3784-92.
253. Jung B, Bharadwaj R, Santiago JG (2006) On-chip millionfold sample stacking using transient isotachophoresis. Anal Chem 78:2319-27.
254. Lichtenberg J, Verpoorte E, de Rooij NF (2001) Sample preconcentration by field amplification stacking for microchip-based capillary electrophoresis. Electrophoresis 22:258-71.
255. Gong M, Wehmeyer KR, Limbach PA, Arias F, Heineman WR (2006) On-line sample preconcentration using field-amplified stacking injection in microchip capillary electrophoresis. Anal Chem 78:3730-7.
256. Simpson PC, Roach D, Woolley AT, Thorsen T, Johnston R, Sensabaugh GF, Mathies RA (1998) High-throughput genetic analysis using microfabricated 96-sample capillary array electrophoresis microplates. Proc Natl Acad Sci U S A 95:2256-61.

257. Cheng J, Waters LC, Fortina P, Hvichia G, Jacobson SC, Ramsey JM, Kricka LJ, Wilding P (1998) Degenerate oligonucleotide primed-polymerase chain reaction and capillary electrophoretic analysis of human DNA on microchip-based devices. Anal Biochem 257:101-106.

258. Schmalzing D, Koutny L, Adourian A, Belgrader P, Matsudaira P, Ehrlich D (1997) DNA typing in thirty seconds with a microfabricated device. Proc Natl Acad Sci USA 94:10273-10278.
259. Emrich CA, Tian H, Medintz IL, Mathies RA (2002) Microfabricated 384-lane capillary array electrophoresis bioanalyzer for ultrahigh-throughput genetic analysis. Anal Chem 74:5076-83.
260. Woolley AT, Mathies RA (1994) Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. Proc Natl Acad Sci U S A 91:11348-52.
261. Medintz IL, Paegel BM, Mathies RA (2001) Microfabricated capillary array electrophoresis DNA analysis systems. J Chromatogr A 924:265-70.
262. Paegel BM, Emrich CA, Wedemayer GJ, Scherer JR, Mathies RA (2002) High throughput DNA sequencing with a microfabricated 96-lane capillary array electrophoresis bioprocessor. Proc Natl Acad Sci U S A 99:574-9.
263. Salas-Solano O, Schmalzing D, Koutny L, Buonocore S, Adourian A, Matsudaira P, Ehrlich D (2000) Optimization of high-performance DNA sequencing on short microfabricated electrophoretic devices. Anal Chem 72:3129-37.
264. Lin YW, Chang HT (2005) Modification of poly(methyl methacrylate) microchannels for highly efficient and reproducible electrophoretic separations of double-stranded DNA. J Chromatogr A.1073:191-9.
265. Dang F, Tabata O, Kurokawa M, Ewis AA, Zhang L, Yamaoka Y, Shinohara S, Shinohara Y, Ishikawa M, Baba Y (2005) High-performance genetic analysis on microfabricated capillary array electrophoresis plastic chips fabricated by injection molding. Anal Chem 77:2140-6.
266. Zhou XM, Dai ZP, Liu X, Luo Y, Wang H, Lin BC (2005) Modification of a poly(methyl methacrylate) injection-molded microchip and its use for high performance analysis of DNA. J Sep Sci 28:225-33.
267. Ricco AJ, Boone TD, Fan ZH, Gibbons I, Matray T, Singh S, Tan H, Tian T, Williams SJ (2002) Application of disposable plastic microfluidic device arrays with customized chemistries to multiplexed biochemical assays. Biochem Soc Trans 30:73-8.
268. Llopis SD, Stryjewski W, Soper SA (2004) Near-infrared time-resolved fluorescence lifetime determinations in poly(methylmethacrylate) microchip electrophoresis devices. Electrophoresis 25:3810-9.
269. Pumera M (2006) Analysis of nerve agents using capillary electrophoresis and laboratory-on-a-chip technology. J Chromatogr A 1113:5-13.
270. Wang J, Zima J, Lawrence NS, Chatrathi MP, Mulchandani A, Collins GE (2004) Microchip capillary electrophoresis with electrochemical detection of thiol-containing degradation products of V-type nerve agents. Anal Chem 76:4721-6.

271. Fruetel JA, Renzi RF, Vandernoot VA, Stamps J, Horn BA, West JA, Ferko S, Crocker R, Bailey CG, Arnold D, Wiedenman B, Choi WY, Yee D, Shokair I, Hasselbrink E, Paul P, Rakestraw D, Padgen D (2005) Microchip separations of protein biotoxins using an integrated hand-held device. Electrophoresis 26:1144-54.
272. Renzi RF, Stamps J, Horn BA, Ferko S, Vandernoot VA, West JA, Crocker R, Wiedenman B, Yee D, Fruetel JA (2005) Hand-held microanalytical instrument for chip-based electrophoretic separations of proteins. Anal Chem 77:435-4.
273. Woolley AT, Hadley D, Landre P, deMello AJ, Mathies RA, Northrup MA (1996) Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device. Anal Chem 68:4081-6.
274. Swerdlow H, Jones BJ, Wittwer CT (1997) Fully automated DNA reaction and analysis in a fluidic capillary instrument. Anal Chem 69:848-55.
275. He Y, Zhang YH, Yeung ES (2001) Capillary-based fully integrated and automated system for nanoliter polymerase chain reaction analysis directly from cheek cells. J Chromatogr A 924:271-84.
276. Zhang Y, He Y, Yeung ES (2001) High-throughput polymerase chain reaction analysis of clinical samples by capillary electrophoresis with UV detection. Electrophoresis 22:2296-302.
277. Zhang N, Yeung ES (1998) On-line coupling of polymerase chain reaction and capillary electrophoresis for automatic DNA typing and HIV-1 diagnosis. J Chromatogr B 714:3-11.
278 Hashimoto M, He Y, Yeung ES (2003) On-line integration of PCR and cycle sequencing in capillaries: from human genomic DNA directly to called bases. Nucleic Acids Res 31:e41.
279. Burns MA, Johnson BN, Brahmasandra SN, Handique K, Webster JR, Krishnan M, Sammarco TS, Man PM, Jones D, Heldsinger D, Mastrangelo CH, Burke DT (1998) An integrated nanoliter DNA analysis device. Science 282:484-7.
280. Lagally ET, Emrich CA, Mathies RA (2001) Fully integrated PCR-capillary electrophoresis microsystem for DNA analysis. Lab Chip 1:102-7.
281. Lagally ET, Scherer JR, Blazej RG, Toriello NM, Diep BA, Ramchandani M, Sensabaugh GF, Riley LW, Mathies RA (2004) Integrated portable genetic analysis microsystem for pathogen/infectious disease detection. Anal Chem 76:3162-70.
282. Lagally ET, Medintz I, Mathies RA (2001) Single-molecule DNA amplification and analysis in an integrated microfluidic device. Analytical Chemistry 73:565-570.
283. Blazej R, Kumaresan P, Mathies RA (2006) Microfabricated bioprocessor for integrated nanoliter-scale Sanger DNA sequencing. PNAS 103: 7240–45.
284. Easley CJ, Karlinsey JM, Landers JP (2006) On-chip pressure injection for integration of infrared-mediated DNA amplification with electrophoretic separation. Lab Chip 6:601-10.
285. Easley CJ, Karlinsey JM, Bienvenue JM, Legendre LA, Roper MG, Feldman SH, Hughes MA, Hewlett EL, Merkel TJ, Ferrance JP, Landers JP (2006) A

fully integrated microfluidic genetic analysis system with sample-in-answer-out capability. Proc Natl Acad Sci U S A 103:19272-7.
286. (2006) "Nucleic Acid-Based Technologies Ramp Up Alternative Methods for Amplifying and Detecting Specific DNA Sequences," Genetic Engineering News. 26(12).
287. Chan EY, Goncalves NM, Haeusler RA, Hatch AJ, Larson JW, Maletta AM, Yantz GR, Carstea ED, Fuchs M, Wong GG, Gullans SR, Gilmanshin R (2004) DNA mapping using microfluidic stretching and single-molecule detection of fluorescent site-specific tags. Genome Res 14:1137-46.
288. Grover WH, Mathies RA (2005) An integrated microfluidic processor for single nucleotide polymorphism-based DNA computing. Lab Chip 5:1033-40.
289. Ohman O, Jacobson G, Ekstrom B (1993) Miniaturized electrophoresis in planar foramtas produced by semiconductor fabrication techniques. In: Program and abstracts ICES-ELPHO 93. Sandefjord, Norway, June 2-4, 1993:24.