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Micro-fluidic (Lab-on-the-Chip) PCR Array Cartridge for Biological Screening in a Hand Held Device:

Final Report for CRADA No 264. PNNL-T2-258-RU With CombiMatrix Corp

EI Rainina

October 2010

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PNNL-T2-258-RU With CombiMatrix Corp, Mukilteo WA

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Pacific Northwest National Laboratory
Richland, Washington 99352

Summary

The Pacific Northwest National Laboratory (PNNL) joined CombiMatrix Corp (CM in Mukilteo, Washington) and the Engelhard Institute of Molecular Biology (EIMB, in Moscow, Russia) in an effort to develop a micro-fluidic cartridge that incorporates sample preparation/nucleic acids extraction, nucleic acid amplification, and microarray analysis as a new solution for accurate, highly sensitive bioagent detection. The goal was to develop a prototype of a "lab-on-the-chip" detector that would enable autonomic processing, and allow identification of bioagents in medicinal and environmental samples.

The project goal was to create integrated disposable cartridges that can be manufactured, complete with all reagents, for less than $35 apiece. The cartridges must support sample preparation, nucleic acid amplification, multiplex genotyping, and detection. Instrumentation to support cartridge operation, nucleic acid hybridization and detection should be small, rugged, affordable, and easy to use, and contain software to interface with a laptop personal computer (PC) for data analysis.

Project partners predicted a high probability of commercial success for this project, because of the lack of any comparable technology on the commercial market, and the perfectly matched expertise, interests, and capabilities of EIMB, CM, and PNNL.

Although this project showed great promise, at the request of EIMB, it was discontinued before reaching completion. Of the 26 milestones identified in the Project Plan, only 18 were completed and shared with the U.S. project partners. Some additional deliverables were at least partially completed; however, EIMB has not been able to share additional findings with CM.

Major results accomplished through the project can be split in three major categories:

- development of the protocol for bioagents concentration within a micro-fluidic cartridge followed by onsite extraction and purification of nucleic acids
- engineering design and prototyping of disposable microfludic cartridge and a platform operating the processes within a cartridge
- software to interface operating platform.

Additionally, extensive experimental work was conducted at EIMB to develop the protocols for efficient nucleic acids extraction from small number of cells and viruses. These protocols were used for a development of "in-cartridge" bioagents concentrating, nucleic acids extraction, and purification.

Acronyms and Abbreviations

CM	CombiMatrix Corp, Mukilteo, Washington
DNA	deoxyribonucleic acid
EIMB	Engelhard Institute of Molecular Biology, Moscow, Russia
PCR	polymerase chain reaction
PNNL	Pacific Northwest National Laboratory
RNA	ribonucleic acid

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1.0 Introduction

The worldwide emergence of new and old has, and will continue to place stress on both medical and clinical diagnostics. The additional potential threat causing by highly infective and stable pathogens such as spores also requires innovative approaches for time-reasonable identification of pathogens. Currently, many, if not most non-typical disease incidence take 5 to 14 days for clinical diagnostics using a variety of fast single-pathogen diagnostics, or very lengthy classical culture methods. Solutions to these and other related diagnostics problems (human health/genetic, environmental, veterinary, food and airborne infections) can only be resolved by a new generation of diagnostics that is robust and rapid.

1.1 Background

The classical approach to bioagent detection involves the use of differential metabolic assays to determine species type in the case of most bacteria, or the use of cell culture and electron microscopy to diagnose viruses and some bacteria that are intracellular parasites. Samples taken from the environment, such as soil and water, and most clinical samples must be cultured in order to obtain sufficient numbers of various cell types for reliable identification. The time required for microbial outgrowth is typically 4 to 48 hours (or two weeks for certain cases, such as *Mycobacterium tuberculosis*).

Using bacterial culture for analysis has other inherent drawbacks; for example: cells that are viable may not be cultured, because they possess unanticipated nutritional requirements as a result of genetic mutation or modification. Biodetection is a very large and active field, which merits a study all by itself.

It is a common opinion that detection of biological agents is a two-stage process involving a probe and a transducer. Probe technology concerns how the assay or detection device recognizes the particular target microbe. Transducer technology concerns how the assay or detection device communicates the activity of the probe to the observer. Together, probe and transduction systems determine specificity, sensitivity, and time required to identify the biological agent.

Nucleic acid-based probes capitalize on the extreme selectivity of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) recognition. Nucleic acid probes—engineered single strands of RNA or DNA—bind specifically to strands of complementary nucleic acids from pathogens. These probes and their binding can be detected directly or by tagging with an easily detected molecule that provides a signal. The design of the probe can be highly specific if there is a good fit to a pathogen-unique region of the target nucleic acid, or it can provide more generic identification if there is a fit with a region of nucleic acids conserved among several related pathogens. The sensitivity of these hybridization assays for bacteria is between 1,000 and 10,000 colony-forming units; improved sensitivity is an important area of research. Since the reaction is in real time, the time-consuming part of the method relates to sample preparation and the time required to detect the signal.

The main advantages of nucleic acid-based methods are

- universality (all living organisms have DNA and/or RNA)
- specificity (every type of organism has some unique sections of DNA or RNA)

- sensitivity (with amplification, very small amounts can be detected)
- adaptability (base sequences common to several microbes, or even a whole class of microbes, can be used as probes)
- multiplex capabilities for a host of different microbes (a sample can be probed for many different sequences simultaneously).

Disadvantages include difficulty in isolation and "clean up" of DNA samples, degradation of the nucleic acid probes, and interference from related sequences or products. These are important obstacles to be overcome, even after specific and accessible target sequences are identified and probes constructed.

In general, degradation of the nucleic acid probes and interference from related sequences or products from the microbial environment significantly limit current applications of this technology beyond well-equipped and experienced laboratories. A single microbial cell can be detected in the laboratory from highly purified DNA by these methods, but environmental samples have regularly failed to achieve this, usually having a detection limit of 105 microbes. A polymerase chain reaction (PCR) assay detected 100 percent of spiked samples in one study but only 15 percent of naturally infected samples.

The long-term goal in bioagent detection is to develop a hand-held instrument featuring disposable cartridges that contain all the necessary reagents, reaction chambers, waste chambers, and micro-fluidics to extract, concentrate, amplify, and analyze nucleic acids. This unit would be able to provide real-time analysis at high sensitivity levels and with low levels of false positives/negatives. The current field-portable detection technology uses immunoassay technology combined with chromatographic transduction. These units have a low sensitivity compared to clinical laboratory testing, and are prone to high levels of false positives/negatives. However, an advanced sensing device could theoretically be built on a DNA/RNA-based technology platform. The market for such devices was estimated at over $1 billion.

2.0 Project Goal

The Pacific Northwest National Laboratory (PNNL) joined CombiMatrix Corp (CM in Mukilteo, Washington) and the Engelhard Institute of Molecular Biology (EIMB, in Moscow, Russia) in an effort to develop a micro-fluidic cartridge that incorporates sample preparation/nucleic acids extraction, nucleic acid amplification, and microarray analysis as a new solution for accurate, highly sensitive bioagent detection. The goal was to develop a prototype of a "lab-on-the-chip" detector which would enable autonomic processing, and allow identification of bioagents in medicinal and environmental samples.

Microarray technology has been developed for a number of years by the EIMB and spin-off company, Biochip-IMB, Ltd., in Moscow, Russia. The biochips produced by EIMB/Biochip-IMB, called IMAGE (immobilized micro array of gel elements) chips, are microarrays of semispherical hydrogel pads with a size less than 80 μm in diameter and less than 0.2 nanoliter in volume. The gel pads contain chemically immobilized compounds, such as oligonucleotides, DNAs, proteins, antibodies, and other probes and supporting chemistries. The use of the three- dimensional gel pads provides several essential advantages including a much higher capacity for immobilization, and a homogenous, water-based surrounding environment. The array's unique properties could also use the gel pads as nanoliter-test tubes for a variety of processes (e.g., DNA hybridizations, chemical and enzymatic reactions such as mini-sequencing and PCR amplifications, etc.).

The current third-generation IMAGE chip manufacturing process provides a fast, reproducible, and inexpensive means for producing IMAGE chips of varying complexities. Individual chips may contain one hundred to several thousands of gel-pad immobilized compounds each. Hybridization of oligonucleotide microchips, such as the IMAGE chip, with PCR-amplified DNA or RNA and different genes has been successfully applied for reliable and fast identification of many bacteria and viruses. An efficient diagnostic approach has been developed, successfully tested, and certified by the Russian Health Ministry to detect *M. tuberculosis* and its mutations responsible for drug-resistance. The results were reported in many papers and patented.

CM also designed and built an automated synthesizer with controlling software to mass-produce customized gene arrays along with software algorithms to efficiently design oligonucleotide probes for hybridization experiments. This work resulted in an automated system for designing probes to meet individual user requirements for genotyping and gene expression analysis. Probe design files are ported to the synthesizer where a single or multiple arrays can be produced within 24 hours. Array designs can be further modified with simple keystroke entries into design files and new arrays produced without incurring setup or modification costs. This versatility allows users to iteratively build, test and modify custom microarrays with a minimum up-front investment.

CM believed that, in conjunction with EIMB, it would be possible to develop a small, integrated, automated system with user-defined capabilities that optimize requirements for genetic information against the cost to collect that information. CM and EIMB also proposed to collaborate in areas of on assay development, bioinformatics, and molecular biology to develop new approaches and assays for diagnostics and disease surveillance.

The project goal was to create integrated disposable cartridges that can be manufactured, complete with all reagents, for less than $35 apiece. The cartridges must support sample preparation, nucleic acid

amplification, multiplex genotyping, and detection. Instrumentation to support cartridge operation, nucleic acid hybridization and detection should be small, rugged, affordable, and easy to use, and contain software to interface with a laptop personal computer (PC) for data analysis.

Project partners predicted a high probability of commercial success for this project, because of the lack of any comparable technology on the commercial market, and the perfectly matched expertise, interests, and capabilities of EIMB, CM, and PNNL.

3.0 Project Plan

The project concentrated the efforts on three distinct complementary elements: (1) micro-fluidic disposable pre-packed cartridge; (2) autonomic operational unit/platform; (3) user friendly software allowing DNA/RNA extraction followed by amplification of nucleic acids and automatic detection.

The goal would be for the user to collect the sample, inject the raw sample into the cartridge, insert the cartridge into platform (which automatically supports micro-fluidics within the cartridge), and then press the "start" button. This automated process flow would protect the sample from contamination and minimize the user's exposure to the sample. The sample would entirely contained within the cartridge, so that the analysis unit would be reusable, while the cartridge would be appropriately disposed as biological material or hazardous waste, or sent to a laboratory for confirmatory analysis. Upon completion of the run, the device will display the results of the test in a simple, easy-to-understand format.

4.0 Critical Outcomes of the Project

Although this project showed great promise, at the request of EIMB, it was discontinued before reaching completion. Of the 26 milestones identified in the Project Plan, only 18 were completed and shared with the U.S. project partners. Some additional deliverables were at least partially completed; however, EIMB has not been able to share additional findings with CM.

Major results accomplished through the project can be split in three major categories:

- development of the protocol for bioagents concentration within a micro-fluidic cartridge followed by onsite extraction and purification of nucleic acids
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Additionally, extensive experimental work was conducted at EIMB to develop the protocols for efficient nucleic acids extraction from small number of cells and viruses. These protocols were used for a development of "in-cartridge" bioagents concentrating, nucleic acids extraction, and purification. The results of the project, accomplished by EIMB team, can be summarized as follows.

1) The concentrating micro-column was prototyped as a part of micro-fluidic cartridge. This micro-column allowed concentration of microbial cells and viruses from low concentration environmental and medicinal samples (e.g., 10 cells/viruses per one milliliter of sample). This concentrating element improved the sensitivity of detection of biopathogens by at least two orders of magnitude from other tools on the market.

2) The lysing buffer was optimized, and the protocol for efficient lysis of bioagents in contaminated samples was developed. Lysis of bioagents in medicinal and environmental samples is a first step in nucleic acid extraction. To a great extent, this stage dictates efficacy of detection and therefore determines the overall sensitivity of a detecting method. The higher efficacy of the lysis, the lower the number of cells/viruses are required to collect the nucleic acids necessary for the subsequent amplification. Development of the lysis protocol contributed to efficacy of bioagents detection.

3) The reagents for nucleic acid isolation/extraction/purification were optimized, resulting in improved efficacy and accuracy of nucleic isolation. As few as 500 of bacterial cells or viral particles in "real world" samples were sufficient for DNA/RNA isolation within micro-fluidic cartridge for further PCR-amplification.

4) The first prototype of micro-fluidic cartridge for DNA/RNA extraction was designed and manufactured from organic glass. Liquid reagents were mixed in optimal proportions and pre-filled inside designated compartments within a cartridge.

5) The first prototype of an operational platform was designed and manufactured for an application with an initial cartridge prototype. Multiple experiments were conducted with platform prototype, and considerations identified for the cartridge redesign.

6) The second prototype of a platform was designed and manufactured, and a second cartridge prototype was designed to complement the new platform. These experiments revealed inconvenience of operating with liquid reagents. It was shown that pre-mixed liquid reagents had low stability and were inconvenient for prepacking a cartridge. In the light of future mass-production of prepacked disposable cartridges and a necessity for their long-term storage, these drawbacks were very significant. Therefore, extensive efforts were focused on a development of solid reagents for bioagents lysis, and nucleic acids extraction and purification. A new method for lyophilization of pre-mixed liquid reagents was developed to protect the reagents against inactivation because of lyophilization, and to obtain solid lyophilized reagents easy soluble with minimal volume of liquid within a cartridge.

7) Stable, solid compositions of pre-mixed reagents (necessary for on-cartridge pathogens lysis and DNA/RNA extraction and purification) were developed, allowing for appropriate shelf stability of the cartridges.

8) The protocol for DNA/RNA isolation and purification from bacterial cells and plasma samples containing viral particles was developed and integrated into a micro-fluidic device prototype.

9) An extensive material compatibility study was conducted to choose appropriate materials for final cartridge design and manufacturing. Materials were chosen for study that

- would not compromise biochemical reactions ongoing within a cartridge
- were compatible with RNA/DNA
- had minimal to no absorption characteristics.

Consideration was also given to cost of the material. Several appropriate materials were suggested by EIMB group and CM.

10) Two models of a reusable cartridge for nucleic acid isolation have been manufactured. The first version was a “two-dimensional” model where all compartments and reservoirs were arranged in plane. A “three-dimensional” prototype of disposable cartridge was also manufactured. This version of an experimental non-disposable cartridge was considered as a direct prototype of a disposable micro-fluidic cartridge.

11) Two versions of the software have been designed to operate two prototypes of the disposable micro-fluidic cartridge. Both versions of the software use USB or Ethernet ports, which allow use of the software without any additional drivers:

- “Two-dimensional ” software allowed optimizing many parameters of analysis, including necessary pressure, management of air and fluid valves, mixing, processing time for each stage, and heating of individual reservoirs up to a predetermined temperature. A special algorithm for heating was designed.
- “Three-dimensional (3D)” software operates the 3D cartridge prototype directly associated with an operational platform. The 3D prototype enables operator to control on-cartridge DNA/RNA isolation either manually or in an automatic mode.

12) CM and EIMB specialists agreed to manufacture disposable cartridges at CM facility. CM technical staff and engineers were identified to work with EIMB scientists and engineers.

5.0 Perception of the Commercial Value of the Project

This project had diverse aspects that were and are important to Russian and U.S. interests, and there is an extensive and growing market for inexpensive, user-friendly, reliable, and durable systems for pathogen detection. The integration of EIMB's scientific expertise and pre-existing technology with CM's experience and resources in microarray manufacturing could lead to the development of a novel system for automatic extraction/amplification of nucleic acids. Partners assessed the project as having a moderate risk level, because of the development of a novel technology. The risk level was considered appropriate, in light of the potential project outcomes.

CM identified a range of markets in a wide variety of government and commercial sectors, and targeted the following list of markets as potential adopters of a novel, standardized, easy-to-use, low-cost detection system:

- Biodefense and Homeland Security for monitoring biothreat agents, emerging infectious diseases, and for first responders ($10 billion U.S. market)
- Human diagnostics molecular testing, immunodiagnostics, and point of care ($6.4 billion worldwide market)
- Companion animal veterinary for infectious disease diagnostics, cancer diagnostics, etc. ($3.5 billion worldwide market)
- Commercial animal veterinary ($650 million worldwide market)
- Environmental testing ($350 million worldwide market).

6.0 Plans for Future Commercialization

Without the remaining critical project deliverables, CM does not have the technology or the experimental data to pursue commercialization.

7.0 List of Inventions Developed Based on this Work

There were no inventions by the U.S. or EIMB teams at the time of this report.

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