1. Executive Summary - Clinically Significant Concern Addressed and Intended Use of the Proposed Device

The emergence of antibiotic resistance in bacteria presents an enormous challenge since the treatment of many bacterial infections using available antibiotics is becoming increasingly limited, and in some cases, nonexistent. Based on CDC reports, 2 million people acquire antibiotic-resistant infections each year and about 23,000 patients die[1]. Recently, an alarming increase of various types of antimicrobial-resistant gram-negative bacteria is observed[2], even in children hospitalizations[3], including extended-spectrum b-lactamase (ESBL)–producing *Enterobacteriaceae*, carbapenem-resistant *Enterobacteriaceae (*CRE), and multidrug-resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[4] Healthcare-associated infections (HAI) complicate over 10% of all hospitalizations and up to 75% of those hospitalizations are caused by pathogenic bacteria that are resistant today to the first-line of antimicrobial therapy. This significantly increases morbidity and mortality in HAI and increased annual healthcare costs in US to over $20 billion. Timely and rapid diagnostics of bacterial infections and recognition of their resistance toward specific antibiotics is becoming a critical step in implementing measures to reduce unnecessary or ineffective prescriptions of antibiotics. A practice of frequently prescribing antibiotics, when they may not be needed, promotes evolutionary or selective pressures toward the development of new strains of antibiotic resistance bacteria. DNA-based, molecular diagnostics technologies provide a finger-print type identification of both bacterial species and their resistance genes. This project proposes development of a ***new POC DNA microarray platform*** and an ***assay correlating the resistance genes with antibiotics susceptibility*** for ***outpatient applications, that will target clinics, emergency rooms, urgent care centers*** and ***physician offices.***

2. Description of the Proposed Point-of-care DNA Microarray Technology for Outpatient Applications

Current point-of-care (POC) DNA based technologies that can be used to accurately identify bacterial pathogen infections **do not provide**:

(i) A ***satisfactory time of analysis***; today commonly within 1-2 hours; a satisfactory time for the sample-to-answer analysis in the outpatient settings should be within the time while the patient is still under examination, thus ~25-30 min, or less (resembling a Strep Throat test)

(ii) ***Required levels of multiplexed pathogen detection***; the current methods predominantly use real-time PCR detection that is limited by a number of colors used in the optical detection, for instance 4-6 (e.g., Cepheid's and Roche's Iquum systems); the only commercialized POC microarray-based assays (BioMerieux's FilmArray and BCID Panel, shows up to 20-25 pathogens/genes, however it is not focused on the prescription of antibiotics, and does not address the real problem with the detection of bacteria: a need to cover a much larger variety of pathogens and resistance genes to be able to provide a correlation with the use of antibiotics. The number of critical pathogens, listed in this solicitation is 18, and considering multiple genes (2-3) for their identification, and covering a broader range of main resistance genes, say 40-50, would require a detection of at least 50-100 analytes;

(iii) ***Satisfactory cost –*** current DNA POC methods on the market ***have too high cost*** of a disposable cartridge and instrument to satisfy the outpatient markets.

The proposed Nexogen's DNA POC microarray platform is based on several innovations that will offer the following ***comparative advantages***:

(i) Simple, automated system that will target ***CLIA-waived level*** of complexity for the test and instrument:

(ii) ***No-moving parts microfluidics*** that assures: 1. containment of all fluids within the disposable cartridge; 2. automated transport of sample and reagents within the cartridge; 3. no liquids or infectious samples within, or in contact with the instrument components; 4. all pumping mechanisms are embedded within the disposable cartridge, thus no fluidics moving part in the instrument; 5. Very simple, low cost instrument design with minimum mechanical components.

(iii) An extremely rapid, and efficient ***sample preparation*** system that combines chemical, enzymatic and mechanical lysis capable of ***6-8 minutes*** DNA extraction with the efficiency equivalent to Quiagen Mini Prep kits

(iv) An extremely rapid isothermal target amplification enhanced by ***convection fluidics*** that enables rapid, ***10-15 minutes amplification*** of DNA/RNA targets

(v) An ***array based amplification and fluorescence detection*** that assures very high levels of multiplexing compared to current POC DNA systems on the market, in the range: ***50-600 pathogens / resistance genes***

(vi) Assay data will improve the patient management by: (i) detecting presence of bacteria and need for administering antibiotics: (ii) based on a broad range of resistance genes screen – a ***recommendation*** will be given to which antibiotics the detected pathogens are resistant and/or ***which antibiotics to prescribe***

(vii) ***Low cost cartridge*** production using simple plastics molding and screen printing processes

(viii) modified SLR camera and fluorescence optics for a low cost, and ***sensitive fluorescence array detection*** of DNA/RNA targets on the array.

(ix) Simple, ***low cost, portable instrument design*** for single sample analysis, with the ***sample-to-answer time in the range 20-25 minutes***.

(x) Software including simple graphical user interface with embedded controls of the process and LIS-type (Laboratory Information System) connectivity to health-care systems

The proposed Nexogen's POC platform will implement the following ***innovations*** to achieve the system and assay characteristics summarized above:

(i) ***No-moving parts microfluidics*** is based on a proprietary pumping and valving system using ***electrolytically generated, pressure balanced pumping*** system within the cartridge that requires no external mechanical devices.

(ii) A ***magnetic beads-based DNA extraction*** with a proprietary sample preparation that combines chemical, enzymatic and mechanical lysis assuring ***6-8 minutes*** DNA extraction with high efficiency

(iii) An extremely rapid ***rolling circle amplification isothermal*** target amplification enhanced by ***convection fluidics*** capable of ***10-15 minutes amplification*** of DNA/RNA targets

(iv) An array based amplification and fluorescence detection that assures very high levels of multiplexing up to: range 50-600 pathogens / resistance genes

(v) ***Low cost miniaturized cartridge*** production using simple plastics molding and ***screen printed electrolytic pumps and detection array***

(vi) Low cost modified SLR camera and optics enables ***sensitive fluorescence array detection***.

(vii) Simple, ***low cost, portable instrument design*** for single sample analysis, with the ***sample-to-answer time in the range 20-25 minutes***.

(viii) Easily modifiable software based on National Instruments' miniature electronics components (miniature field programmable array – FPGA) and Labview programming language

2.1. The Required Specifications for the Outpatient Diagnostic Application

Ninety percent of hospitals in the U.S. and worldwide cannot perform DNA/RNA based analysis for identification of infectious, pandemic or emerging bacterial or viral diseases and samples are sent to

reference labs, to be returned in 2-3 days. Because of this delay, no adequate prescription of antibiotics can be made. Appearance of emerging, pandemic infectious diseases would be best recognized if identification could be made at outpatient facilities such as urgent care clinics, Minute clinics, or emergency rooms. However, three major problems with the current DNA POC platforms are remaining unresolved: (i) analysis time should be about 3 times shorter; (ii) cost is unacceptable to those smaller facilities and should be decreased ca 5 x to become of interest to directors and doctor's leading those facilities, and (iii) number of bacteria and their genes is much larger than what the current platforms can detect. Those should include ca 18 bacterial pathogens (from the CDC list and White House Action Plan) [5,6] x ca. 2 genes on average for identification = 36 analytes. According to the ARDB (Antibiotic Resistance Data Base)[7] and CARD[8] (Comprehensive Antibiotic Resistance Database) databases there are over 300 types of resistance genes and over 200 antibiotics. The only array POC technology on the market, the Biomerieux's Film Array looks into only 3 resistance genes, and a range of bacteria that is limited to ca. 20, with over 1h of analysis time and a cost of ~$1,600/sample. Charging $1,600 per sample for determination which antibiotic to recommend, when the cost of the antibiotic use, without insurance coverage, on average is ~ $250 - $300, cannot compete in the Antibiotic Resistance Gene diagnostics market. Sequencing methods could provide a screening of an entire panel and/or a large number of resistance genes, however, today, and most probably for a number of years, the sequencing methods are not close to their use in a point-of-care analysis, and are limited by the unacceptable cost, analysis time, complexity, and/or a requirement for a sophisticated software and need for a long-term software data analysis.

Our vision of the ideal product for Antibiotic Resistance Identification is: a DNA microarray that can screen for a large number of resistance genes, if not all of them, within 15-20 minutes, while the patient is still under examination, and correlate the microarray data with the antibiotics that are pre-screened versus resistance genes. Such an array will provide a clear recommendation which antibiotics would work for a particular pathogen identified in the patient sample. The ***proposed platform can satisfy all those criteria***. Although testing for majority of the resistance genes and recommending among large number of antibiotics, is our ultimate commercialization goal, however, the ***Challenge Project timeline*** may not allow to expand the assay panel to more than 50-80 resistance genes. Our ultimate commercial goal is to develop the platform that can accept up to 400 analytes, i.e., resistance genes that would offer a unique, comprehensive platform for antibiotics treatment recommendations. The advantage of our approach is that the platform will be capable for detection of this large number of analytes as soon as the instrumental platform is developed (~1 year), however expanding the assay panel to such large number of resistance genes will take a longer time-line, and adequate resources (see our Challenge Project and commercial product timeline below).

2.2. Nexogen's Experience in Development of POC Platforms

The Principal investigator and the Nexogen's team pioneered[9,10,11] the electronic microarray technology[12,13,14,15,16,17] for rapid, multiplexed DNA/RNA detection, initially developed at Nanogen and divested and spun-out into Nexogen, Inc., where the technology was integrated with the sample preparation and amplification. Nexogen's initial prototype MDx platform (cf., Fig. 1), is a miniaturized, sample-to-answer system with robotized mechanical fluidics for DNA/RNA diagnostics that packages all analysis steps within a disposable cartridge. The system, CAS-100™, (cf., Fig 1) capable of running 6 cartridges with random access of 3 samples, is in ***commercialized today*** in partnership with Savyon Diagnostics, Ltd and AdorDx, Ltd. (cf., Fig., 1 and 2). The proposing team has over 14 years of multidisciplinary experience in miniaturizing DNA/RNA sample-to-answer analysis systems, as well as POC type assay development on the electronic microarray

Figure 1. Initial Nexogen's sample-to-answer (MDx) single cartridge system (left), for integrated detection of DNA/RNA and a 6 cartridge CAS-100™ system (right), with disposable cartridges for performance of assays where all reagents are contained within the cartridge.

platforms. Two other electronic microarray platforms, targeting a higher sample throughput and reference labs, have been developed in GMP settings: (i) an electronic (400 site) microarray ***detection only***, Nanochip NC400™ system with commercialized assays ranging from cystic fibrosis to respiratory viruses; and, (ii) currently, in collaboration with Savyon Diagnostics[18] and Ador Dx, Nanochip XL™ (includes PCR thermal cycler), and assay panels (BRCA and pharmacogenomics assays, and gastrointestinal panels). In addition to the private funding, the PI's research and development yielded several assay development applications of Nexogen's miniaturized MDx platform where different assay developments were supported through the NIH and Canadian Food Inspection Agency CFIA projects: (i) the carbon array hardware platform was partially supported through a hardware development and CAP/sepsis clinical assays[19,20,21]); (iii) influenza assay subtyping and development[22]; (iii) CFIA supported development of avian influenza, foot-and-mouth disease and high consequence swine and bovine assays[23]. The team was also involved in the miniaturization of detection equipment for biological agent speciation[24,25] and forensic analysis[26].

Nexogen has full access/license arrangements to the electronic microarray patent portfolio (ca 80 patents) through its partnership and IP exchange with Savyon Diagnostics (http://www.savyondiagnostics.com/ (Savyon Diagnostics is a member of Gamida for Life Group of companies, based in Netherlands, which is engaged in Healthcare related operations worldwide) and AdorDx (http://www.adordx.com/ (ADOR Diagnostics is an international IVD company headquartered in Rome, Italy).

Figure 2. Envisioned product Nexi-Dx™ POC Microarray DNA system and cartridge. The simple steps assure that the instrument and assay will not require highly skilled personnel, and will comply with CLIA-waived regulations.

The proposed system and the Antibiotic Resistance Assay (ARA) leverage our previous solutions on the POC platforms and assay development, but represents here ***our next generation platform, that involves further simplifications and improvements that are not involved in the present instrumentation and platforms.***

2.3. The Proposed DNA Microarray POC System for Identification of Antibiotic Resistance – Nexi-Dx™

Figure 2 shows the envisioned product for the DNA Microarray POC Identification of Antibiotic Resistance in outpatient settings, named Nexi-Dx™. Figure 1 demonstrates the simple steps of the analysis that will

warrant CLIA-waived complexity, and will not require high skill for the operation. This is important so as the system and assay could be run by registered nurses, e.g., in urgent care clinics. The steps imply minimization of user errors and a graphical user interface on the touch-screen that will guide the user in the analysis steps: (i) scan the sample; (ii) insert the sample into cartridge, enclose the cartridge by capping it; (iii) scan the cartridge; and (iv) insert the cartridge into the instrument and press run.

The following presents ***innovations*** in more details that will assure the performance of the proposed system and to satisfy the requirements for its implementation in outpatient facilities.

2.3.1 New Microfluidics Electrochemical Balanced Pressure Pumping System

Compared to the instrumentation available on the market, and our previous POC platform (cf., Fig 3), this project introduces significant simplifications in the microfluidics, both in the cartridge design and instrument design and will present the next generation POC platform that will enable a disruptively low cost and rapid sample-to-answer analysis time. Over 15 different reagents, typically used from DNA/RNA sample preparation to amplification and detection, ranging from uL to mL volumes are packaged using a unique, ***proprietary Electrochemical Balanced Pressure (EBP) microfluidics*** *that requires* ***no-moving parts within the cartridge.*** (cf., Fig. 3 and 4) that is based on balancing electrolytically generated pressures in pumps and valves embedded within the simple, easy to mold, block cartridge.

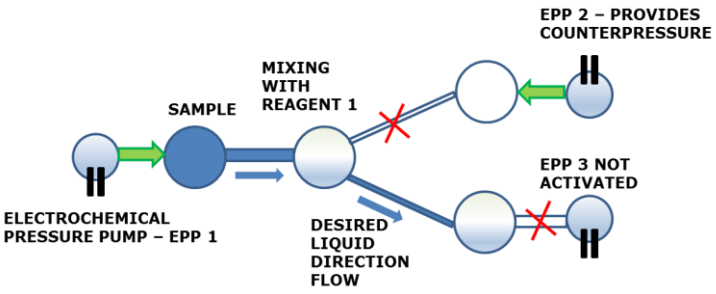


Figure 3. New proprietary Electrochemical Balanced Pressure (EBP) microfluidic principle that enables an extremely simple, but accurate transport of very small volumes, from microliters, to relatively large volumes, milliliters, used when typical clinical samples, e.g., blood are used. Each reagent chamber is connected to one EPP pump, and their easy control enables both valving and pumping actions within the cartridges.

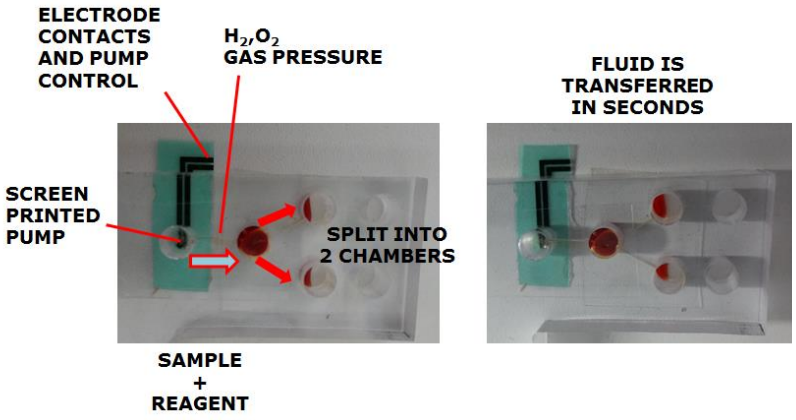


Figure 4. We have performed preliminary simple testing of the concept of Electrochemical Balanced Pressure (EBP) microfluidic pumping principle. The photographs demonstrate simple, accurate and fast pumping between one chamber and pushing or splitting the fluid into two chambers accurately.

Figure 5 shows a detailed schematic of the new EBP fluidics principle for efficiently moving the reagents and samples in the cartridge that guarantees an extremely low cost of manufacturing of the cartridges. A simple principle is based on on-chip wells supplied with screen printed (carbon ink based) electrodes that serve as the miniature pumps containing simple salt solution that enables on-chip pressurization and pumping based on water electrolysis and Faraday's law (cf., Fig. 5). The movement of fluids, and valving is performed by proper adjustment of pressures in the pumps by regulating voltage/current and time of energizing the pump electrodes. In accordance to the Nernst equation, one can demonstrate that extremely high pressures (several hundred psi) can be generated electrochemically, if needed within the fluidic system. By combining the ideal gas and Faraday law one can derive the flow rate and pressures generated within the pump wells:

Flow Rate = (3 RT I / 4F) * ΔV / (PV – P_{atm} V_0)

Where ΔV is the volume of displaced liquid, and V is the volume of the air and the electrolysis gas mixture, equal to V_0 + V. Thus, for generating a pressure of ~ 100 psi, and using a volume of the pump well of ca 150 microliters, one would need only about 0.5 ul of the pump electrolyte. This yields an extremely small dimension of the pumping device of ca 1 mm in diameter and 1 mm well depth. Multiple pumps are used within the cartridge, each reagent chamber connected to its own pump and the fluidic channels connected in such a way that the counter pressures generated in the cartridge direct the fluid movement between the sample, reagent, amplification or waste wells. In this way, the pumps serve also as valves where needed. The combination of pumps and wells enables an open and flexible platform that can easily accommodate all reagents and processes performed within the cartridge, including magnetic bead based separation, e.g., by bringing the magnet or heaters (mounted within the instrument) closer to the sample preparation or amplification chambers, (no thermal cycling needed, heating for enzymatic reactions at ca 45 - 55 C).

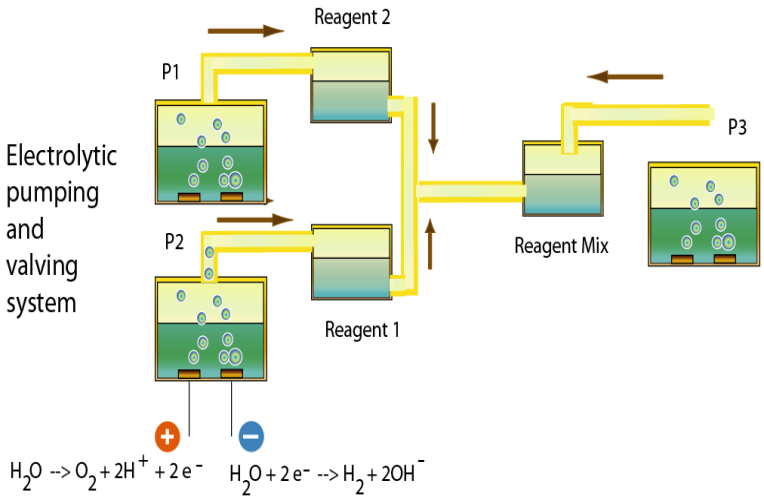


Figure 5. Schematic of the EBP microfluidic principle and operation of the pumps and valves used in the proposed POC platform. The system comprises no moving parts and by combining multiple electrolytic pumping/valving units embedded within the cartridge the reagents and samples are mixed by adjusting the opposing pressures in each pump connected to the reagent/sample reservoirs.

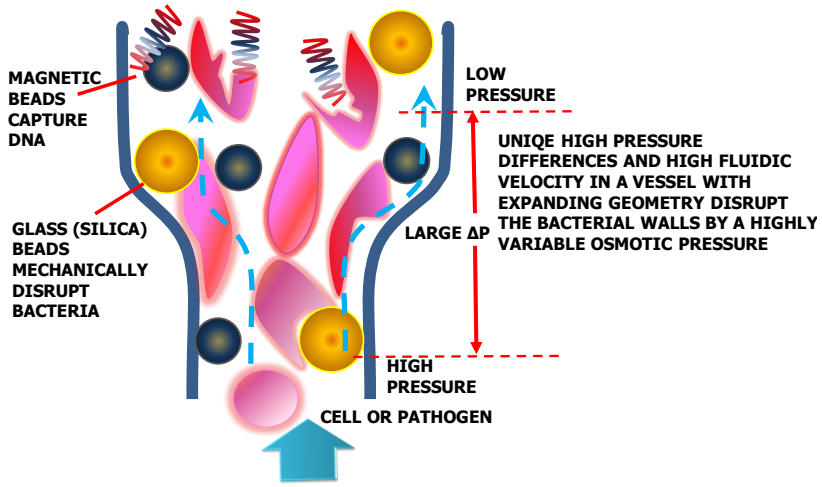


Figure 6. Schematic of the proprietary magnetic beads-based DNA extraction that includes the EBP fluidics, convective mixing and combined chemical, enzymatic and mechanical lysis of target sample.

The cartridge embeds magnetic bead based extraction, extremely rapid isothermal branched RCA amplification, and a novel fluorescence detection using modified, low cost SLR camera optics to yield highly sensitive fluorescence detection on the carbon electronic microarray.

2.3.2. Proprietary Sample Preparation – enables 6-8 minutes DNA extraction

Figure 6 demonstrates the principles of Nexogen's proprietary magnetic beads-based DNA extraction process that includes the EBP fluidics (cf., Fig. 5), enhanced with convective mixing and combined chemical, enzymatic and mechanical lysis of clinical sample. The cartridge has all reagents contained (up to 10-15 different reagents), ranging from uL to mL volumes. The lysis uses chaotropic salts, addition of enzymatic agents (e.g., proteinase K), and mechanical lysis that occurs due to increased shear forces (and/or bead beating), within a narrow and widening channel of the that creates large differences in pressure (under EBP pumping) and fluidic velocity, thus, imposing a highly variable osmotic pressure on the cells, and/or

pathogens in the sample. This causes an extremely rapid cell lysis. In this project, we will leverage our proprietary sample preparation chemistry that consist of the following four solutions: (1) lysis/binding solution (guanidine thiocyanate, lauryl sarcosine, Tris-HCl, isopropanol, agent-coupled magnetic beads); (2) wash I buffer (guanidine thiocyanate, lauryl sarcosine, Tris-HCl, isopropanol; (3) wash II buffer (KCL, EDTA, Tris-HCl, ethanol; (4) elution buffer (TE buffer). Experimental data showing successful, high efficiency DNA extraction from a variety of the sample types are summarized in the Attachment: EXP1-Sample Prep.

2.3.3 Convectively Enhanced Isothermal Rolling Circle Amplification – enables 10-15 minutes DNA Amplification

Figure 6 demonstrates the proposed convective isothermal RCA amplification that is enhanced using an external vibrational stirring motor that accelerates the hybridization of the target to the circular probe and to the capture probe. Both the circular probe and capture probes (acting as the forward biotinylated primers), specific for each pathogen and/or resistance gene of interest, are anchored onto the streptavidin coated gel at the bottom of each array well. This enables high levels of multiplexing, and no multiplex amplification reaction design is needed (as for the PCR amplification) since each amplification occurs spatially separated and only a single gene design is needed per well. The screen-printed array of the dielectric ink formed wells enables large number of array spots to be easily designed and incorporated into the cartridge.

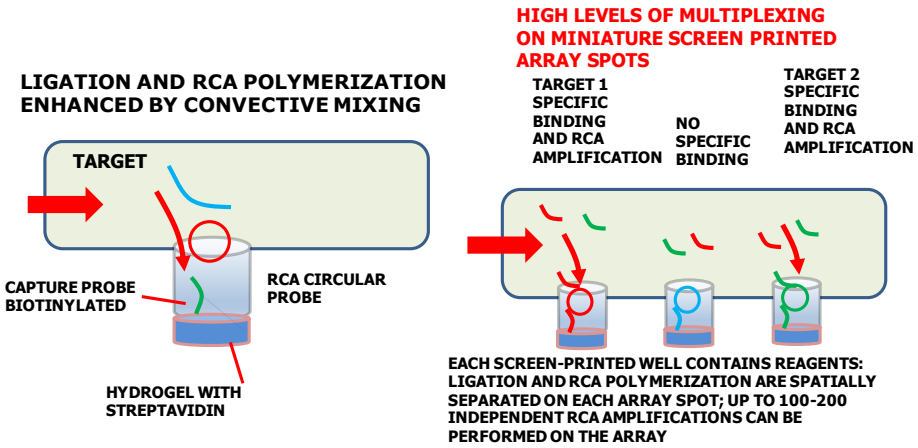
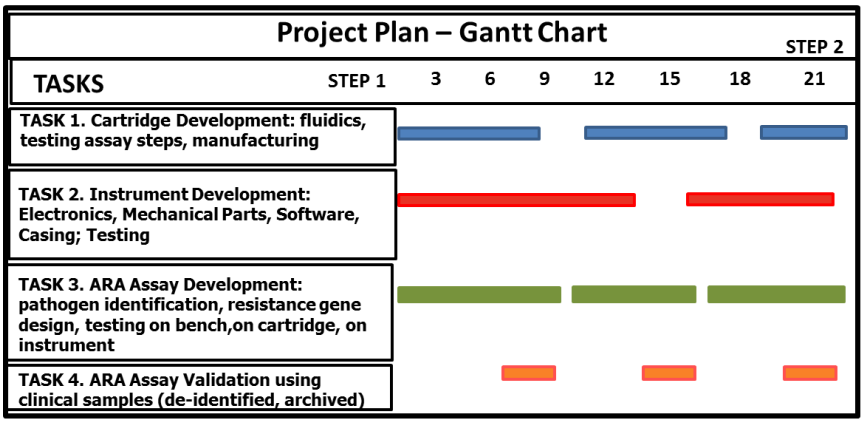


Figure 6: Mechanism of the convection-enhanced isothermal rolling circle amplification. Both ligation and DNA polymerase RCA reactions occur under an external vibrational motor stirring. This enables rapid hybridization of targets, enhances ligation and RCA polymerization. Since the miniature wells are separated, no multiplex design of RCA reaction is needed, and each reaction occurs in its own well. Screen printed array wells, using dielectric ink printing

The cartridge design integrates compartments for the magnetic bead based DNA/RNA extraction, elution of purified nucleic acids into the ligation chamber where the specific circular probes and forward primers are attached and bind to a specific target. The convective RCA is performed in the same chamber by bringing DNA polymerase with strand displacement activity (e.g., NEB polymerase), nucleotides, Mg- salts and appropriate buffer. The RCA amplification enzymes are extremely processive, and yield concatenated products (within 5-15 minutes), by amplifying multiple repeats of the circular probe sequence, anchored to the hydrogel layer. The simplicity of the proposed amplification process is based on the fact that only the 80-120 bp circular probes, specific for a different gene in different wells, are amplified, and not the target itself. Thus, once the process is designed for one well, or resistance gene, it can be easily transferred onto the design of the next resistance gene. The specificity is secured through a double molecular binding mechanism: (i) ligation of the target to the circular probe (highly specific, based on ligation of 2 juxtaposed target sequences on the circular probe, that are about 15 bp each, and (ii) binding of the circular probe to the anchored forward primer. The reverse primer is added with the DNA polymerase reagent solution. The temperatures for ligation and RCA polymerization are relatively low, compared to PCR thermal cycling, and the process does not need cycling of multiple temperatures that results in extremely long times of the amplification process in the PCR reactions. The detection is performed using fluorescence detection of the entire array. We have successfully previously developed a very low cost fluorescence detection that

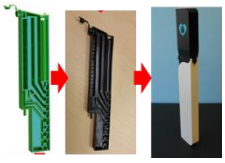
uses SLR cameras and modified optics (see description in Approach Section) for highly sensitive fluorescence detection. We envision that the system will be simple and will include automated self-checks that will enable CLIA certified POC use.



2.4. Approach and Development Plan

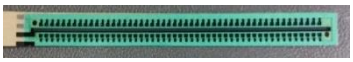
The Project Gantt Chart (left) shows major tasks in the plan for the development of: (i) the instrument platform, (ii) the disposable cartridge, and (iii) the Antibiotic Resistance Array (ARA) assay within the period of 21 month between STEP 1 and STEP 2 submissions. The development of the assay on the platform for the validation in CLIA lab in STEP 3 (2020) will require resolving many of Tasks and issues and will need thorough assay validation throughout the project, and access to adequate bacterial samples/isolates containing the resistance genes. However, based on our previous work, we can leverage a number of solutions for the platform, cartridge, or assay development as well as the manufacturing steps. This will include:

1. Cartridge development:



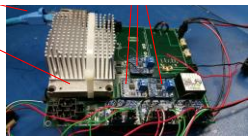
(I) We have determined and tested a range of plastic materials that are compatible with enzymatic reactions, and selected low cost molding processes for rapid manufacturing of the cartridge – photographs show our current CAS-100 cartridge – those will be leveraged in the design of the proposed cartridge.

(II) Screen-printing and array development: we have previously developed an electronic microarray consisting of carbon-based printed inks for electrophoretic transport of analytes – this same technology will be used in manufacturing the proposed microarray wells.



2. Instrument Development:

(i) Major miniaturized electronics components, based on miniaturized, low cost NI FPGA 9651 board, have been incorporated into Nexogen's electronics and accompanying basic software controls are developed in other platforms, and those will be transferred directly to the proposed system



(ii) We have internal capabilities for electronics and mechanical components design and have established collaborators and vendors for manufacturing of mechanical components and cartridge and instrument casings

3. ARA Assay Development: The proposed array platform could offer testing of a large number of antibiotic resistant pathogens and/or their resistance genes, as explained above. A major point relevant to this project is to define a reasonable number of the resistance genes/pathogens that will be included in the proposed ARA assay, and subsequently be reported for the STEP 2 submission, and then validated at n STEP 3 in CLIA labs. Our capabilities are that we have successfully developed in the past multiplex assays of up to 21 genes/SNPs, however this project will require simpler single gene design per the array spot, because of the spatial separation of the wells, which should allow for a higher number of pathogens/genes to be designed and detected. Within the period between STEP 1 and STEP 2, we will therefore propose a limited number of pathogens/resistance genes (that potentially could be modified in that period, depending on the availability of the procured samples) to be validated within the 21 month period. We believe that it will be satisfactory to demonstrate ca. ***15 – 20 pathogen/resistance*** gene detection for the purpose of this project (from STEP1 – STEP3). We

foresee that we will provide a thorough validation of the instrumental platform, cartridges and ARA assay for the ***STEP 2 data*** using clinical (archived, de-identified) ***samples for e.g., 5-7 bacterial pathogens of interest to CDC/NIH/BARDA.*** Preliminary data performed to this point related to development and testing of the assay components and the system are given in the **ATTACHMENT**: (i) sample preparation efficiency using different samples; (ii) Rolling circle amplification performance and detection with antibiotic resistance and other pathogens; (iii) Identification of antibiotic resistance pathogens and genes using PCR and real-time PCR methods – we have developed easy methods that successfully transfer the PCR primer and probe designs into our RCA designs. We will target the following bacteria/resistance genes for the STEP 2:

Table below provides a current Project Organizational Chart and Key Personnel that worked with Nexogen on our previous projects and product development, and will be engaged in the proposed project. Those include: (i) Leading experts in antibiotic resistance: Prof. V. Nizet, UCSD (development of new antibiotics, will provide expertise and access to archived, de-identified (AD) samples; Prof. P. Schreckenberger (University of Loyola, will provide AD samples and independent evaluation of the system and ARA assay); Prof. Miriam Barlow (University of California Merced, AD samples – several hundred b-lactamase isolates and testing); Prof. M. Camps (University of California – expertise in prediction mechanisms for resistance genes; (ii) Institutions that will provide a source of clinical samples (Dr. VonDran, NDRI and Discovery Life Sciences, Emily Dover); (iii) Manufacturing support (Conductive Technologies, Dr. M. Musho – for screen printing; Cartridge and plastics parts molding (Proto-Solutions), machining (ProtoLabs, Leon Fung), Instrument molding (ARRK, John Gabriel), electronics/software, J. Denis; (iv) Marketing support (JantPharmacal) and Commercialization support, R. Cannaviello, Symmetry Advisors, and (v) Regulatory Support – Dr. J. Monforte, WildType Advisors. Nexogen has ***funding support*** from joint partnership Development Agreements with Savyon Diagnostics, LTd and Ador Diagnostics, Ltd. In addition, Nexogen has support through Government Programs funding, e.g., SBIR, where some of the components may be leveraged in the development of this project.

For the submission of ***regulatory*** documents, envisioned in the STEP 3 project, we will engage SynteractHCR, a Clinical Research Organization with whom we worked before, to support the preparation of FDA documentation, validation and submissions. We envision that the submission of our technology to FDA will be based on the ***equivalency with isothermal amplification methods***, that use similar principles, such as the FDA approved Rapid Flu Influenza A and B, a CLIA-waived test by Alere[27].

4. State-of-the Art of current technologies

Figure 7 summarizes a comparison between the proposed technology and the major competitors on the market. Practically all POC technologies on the market are based on real-time PCR that is limited with 4-6 colors, and can perform only a few analytes. Although Roche/Iwuum can perform assays within 25 minutes, they are limited to 4-6 analytes. The only competitive technology on the market, Biomerieux's FilmArray can make up to 20-25 pathogens, but within an hour and at a cost of $1,600 per sample. It is envisioned that the proposed technology will offer the instrument at a cost of $8,000-10,000 and the assay cost will be $50-100 for up to 100 or more pathogens/resistance genes. This presents an improvement of about **15x reduction in the cost per assay, 3x in analysis time, and 10 x in the instrument cost.**

5. Approach to STEP 2

Most of the methodologies planned to be used in the development of the proposed platform and ARA assay are described above.

For the STEP 2 data demonstration and proposal we will have:

1. Instrument built, tested and validate. 2. Cartridge manufactured, tested and validated. ARA assay developed and validated for the following **REPRESENTATIVE** pathogens/genes (we can accommodate to different pathogens if NIH/BARDA prefer to focus on different ones;

Gram-negative pathogens and their representative resistance genes: *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Acinetobacter baumannii; and Pseudomonas Aeruginosa, CRE.*

The choice of the genes characteristic for the antibiotic resistance and relevant to the diagnostic criteria for the above pathogens will be based on the known antibiotic resistance, e.g., Carbapenem resistant *Enterobacteriaceae* (CRE), Extended Spectrum β-Lactamase (ESBL) Producing *Enterobacteriaceae*.

Gram-positive HAI pathogens: *Streptococcus pneumoniae, Methicillin-resistant staphylococcus aureus (*MRSA), *Clostridium difficile,* and Vancomycin-Resistant *Enterococcus.*

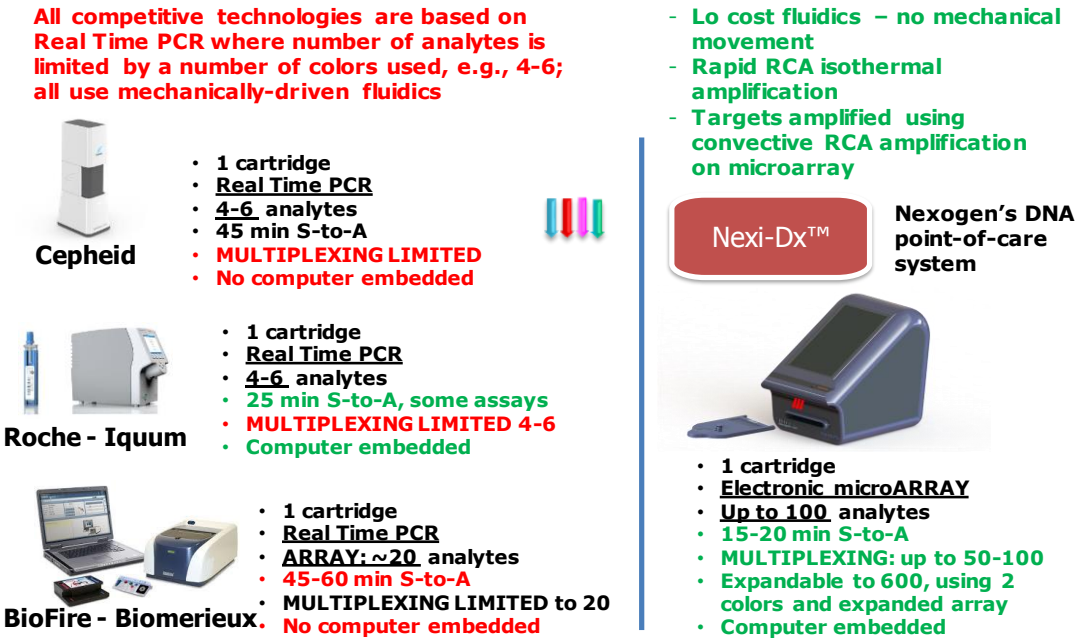


Figure 8: RCA amplification of *E.* coli and initial parameter optimizations: RCA signal on the array vs. amplification time and Mg concentration in the buffer. Successful RCA amplification down to 10 min amplification time was

Nexogen's capabilities are described above, and should include a summary of data performed to date in the Appendix. The table on the left show the Project Organizational Chart and the Key Personnel available to the project.

APPENDIX: EXPERIMENTAL DATA IN SUPPORT OF NEXOGEN PROPOSAL

Assay RCA testing protocols: - Our team, with collaborators from Mt. Sinai School of Medicine previously developed a solution-based real time ramification rolling circle amplification (RAM) using a novel molecular zipper real time probe[28,29,30,31,32]. The isothermal on-chip RCA amplification yields extremely processive amplification within 5-10 minutes. The fluorescence reporting is performed using the dsDNA intercalator dye such as SYBR green or LC green. We have also developed a ***real-time on-chip rolling circle amplification*** (cf., Fig 7.), and successfully demonstrated a 22-plex isothermal amplification on the electronic microarray[33,34], for SNP-based genotyping for human identification. Figure 5 demonstrates correct calls for subtyping of influenza species (H1N1, H5N1, H7N1, H15N1, and H3N1) and using *on-chip*-RCA on the carbon microarray. Table below demonstrates LODs for identification of antibiotic resistance gram-negative pathogens performed using our rapid sample preparation protocols, PCR and carbon array. **This project will not utilize: (i) real-time RCA amplification** – because end detection using red and/or green reporter is satisfactory; (ii) **RCA performed on electronic microarray (cf. Fig 7) -** we have found that RCA reaction performed under convective conditions in small, miniature wells occurs practically within same time as on the electronic microarray.

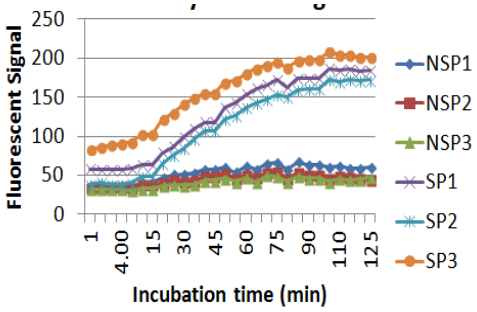


Figure 7: Real-time on-chip RCA curves obtained for titration of H5N1 target on the MDx carbon arrays.

Experimental Data in support of proposed RCA amplification on microarray

Figures below summarize relevant data related to RCA amplification of *E.coli,* as one of the representative antibiotic resistant pathogen.

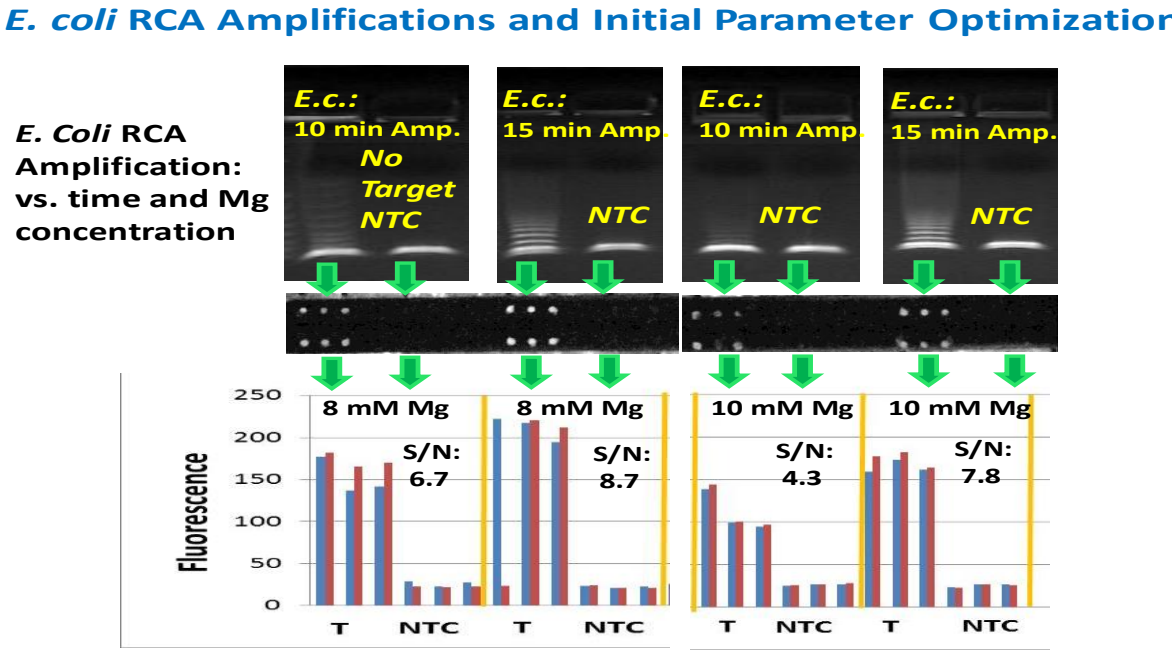


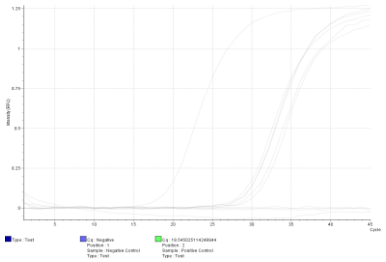
Figure 8: RCA amplification of *E.* coli and initial parameter optimizations: RCA signal on the array vs. amplification time and Mg concentration in the buffer. Successful RCA amplification down to 10 min amplification time was obtained.

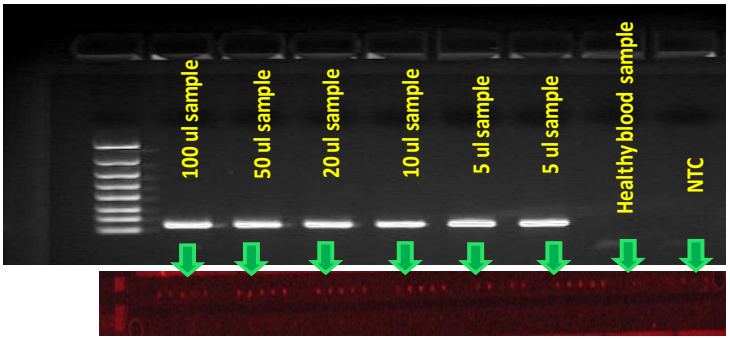
DNA extraction of *E. coli* bacteria spiked into whole blood sample and detection:

1. **Sample preparation time: 6 minutes**
2. **Real-time PCR - Array Detection**
3. **RCA Amplification - array detection**

SAMPLE	Cq	COPY NUMBER
100ul	28.4	1063
50 ul	28.7	802
20 ul	29.2	
10 ul	29.7	427
5ul	30.2	340
Healthy	Negative	

E. coli:
~ 10,000 CFU/ml
Real-time PCR

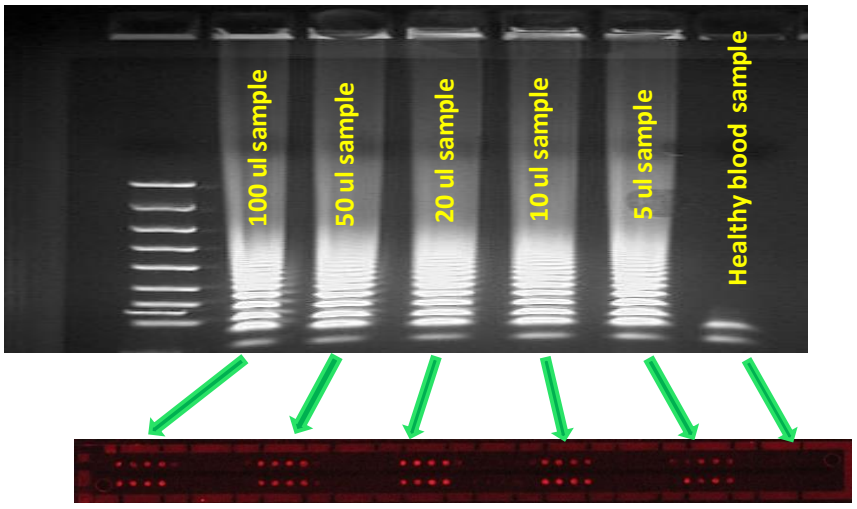




E. coli:
~ 10,000 CFU/ml
PCR probe design and amplification

***E. coli:* Array detection**

Figure 9: Rapid extraction of *E.* coli spiked into whole blood samples and detected using and initial parameter optimizations: RCA signal on the array vs. amplification time and Mg concentration in the buffer: Real-time amplification, and PCR amplification and microarray detection. Dependence on sample volume was measured and satisfactory results were obtained even with 5 microliters sample.



E. coli:
~ 10,000 CFU/ml
RCA probe design and amplification

***E. coli:* Array detection**

Figure 10: Same experiments as in Figure 9 were repeated using our sample preparation and RCA amplification and detection on the microarray. and microarray detection. Dependence on sample volume was measured and satisfactory results were obtained even with 5 microliters sample. Successful RCA amplification was obtained in **15 min.**

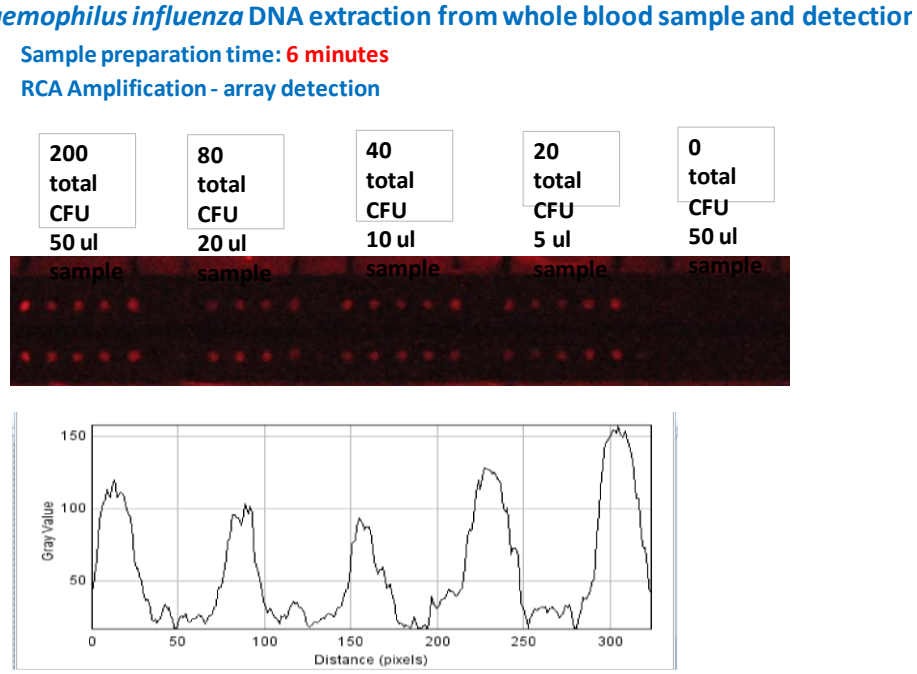


Figure 11: Limit of Detection in the range 20-200 CFU was obtained for H. influenza bacteria spiked into whole blood and at sample volumes ranging from 5 – 50 uL. Sample preparation was performed within 6 minutes, and RCA amplification wtihint 15 minutes.

Nexogen has investigated several methods in the past for sample preparation for both blood and respiratory specimens. For example, we have used antibody-coupled magnetic beads and demonstrated the detection on the NC400/Nanochip platforms for low levels of bacteria spiked into whole blood. We could detect the presence of 100 or fewer organisms/mL of blood using species specific antibodies (Table 1). We have also demonstrated the use of polyspecific antibodies to enrich classes of bacteria. An antibody reported to bind several Gram-positive organisms was found to give good recovery of *S. pneumoniae*, *S. aureus*, *Bacillus subtilis*, and moderate recovery of *Listeria monocytogenes*.

Organism	Detection level
E. coli O157:H7	(per ml blood)
Listeria monocytogenes	25
Streptococcus pneumoniae	100
Staphylococcus aureus	100
Salmonella typhimurium	100
Salmonella typhi	100
Bacillus subtilis	100

Table 1. Detection of organisms purified by Ab-coupled magnetic beads. Bacteria were spiked into human blood, diluted 1:2 with PBS, and then gently rotated with Ab-coupled magnetic beads. After 10-15 min, the beads were collected and washed, and the cells were lysed with heat. An aliquot of the sample was amplified by standard PCR and analyzed either by gel electrophoresis or on the Nanochip 400 System Alternatively, real time PCR was used to quantitate recovery

This sample preparation procedure for the enrichment of bacteria from blood was automated in a prototype device. The first prototype device that does all the steps of sample preparation including mixing, washing, lysis steps, desalting, and detection on the NanoChip 400 electronic microarray has been built, and a prototype was demonstrated and tested at USAMRIID. However, we consider this system too costly, because of the silicon-micro-machined platinum array packaged within the cartridge. Its application is more suitable for the reference lab-applications rather than for the decentralized labs, which is the targeted market for this Phase II project, including emergency, urgent care, and Minute clinics, or doctor's offices as well as smaller and medium hospitals.

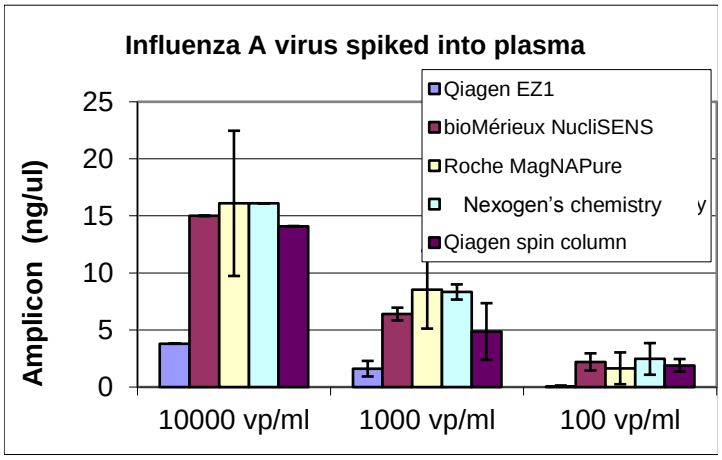


Figure 8. Comparison of different sample preparation methods performed by the proposing team. Influenza virus was spiked into plasma. RNA from the individual aliquots were purified using the methods specified in the legend. The purified RNA was reverse transcribed and amplified by PCR. Capillary electrophoresis was used to determine the concentration of amplicon.

Our proprietary chemistry for sample preparation, using magnetic bead based extraction of DNA was recently developed, and the approach was used in the MDx POC system (cf., Fig 26), for the recovery of viral DNA and RNA from respiratory specimens. This approach and chemistry, that will be further used and/or modified in this project, is based on chaotropic agents for lysis and DNA/RNA preservation. Recovery is achieved by hydrophobic binding on magnetic beads. Recovery obtained was up to 70 to 80% of spiked viral transcripts. Figure 27 shows a comparison of our MDx sample preparation chemistry to several commonly used commercial methods. The data demonstrate that the recovery and cleanliness of the purified nucleic acids compared well with the other methods, cf., Fig 27. This reagent chemistry has been adapted to a cartridge and sample preparation has been done using MDx modules with time for extraction in the range of 15 minutes. Using the MSP system, hardware and chemistry developed in Phase I, we were able to reduce the sample preparation time, for all sample types ***within 8-10 minutes, with 1-2 minutes hands-on time.*** Since there are a few parameters that can be further optimized, and including instrument operation, we expect that the total sample preparation time can be pushed in the Phase II project to ***6-8 minutes***. Compared to Quiagen MiniPrep system, that requires 25-30 minutes, and using more complex and expensive equipment (e.g., a relatively sizeable centrifuge), we consider our approach highly competitive.

Pathogen	Gene	LOD
Influenza A	segment 7: Matrix protein M1	16
Influenza B	NS1 / NS2 genes	1.6
Syncitial virus A	N / NS2 spacer and gene	160
Syncitial virus B	N / NS2 spacer and gene	16
Mycoplasma pneumoniae	P1 adhesion	1.6
Chlamydia pneumoniae	MOMP	1.6
Legionella pneumophila	*mip*	16
Legionella micdadei	*mip*	1.6
Bordetella pertussis	Porin	1.6
Staphylococcus aureus	elongation factor EF-TU	0.016
Streptococcus pneumoniae	pneumolysin *ply*	0.16

Table 2. Example of limit of detection limits (LOD) for our previously developed assays for the community acquired pneumonia and sepsis.

Amplification and detection on Nexogen's POC Systems

Table 2 shows satisfactory limits of detection for the community acquired pneumonia and sepsis assay developed by the proposing team for the Nanochip/NC400 silicon/platinum microarray and platform. Figure 28 demonstrates a successful transfer of this assay, and a sensitive detection of community acquired pneumonia pathogens on the MDx platform that uses a much lower cost, screen-printed carbon electrodes-based array. In another recent study with the CFIA[35], we have developed a multiplex RT-PCR and microarray assay for simultaneous detection of eight viruses that affect cattle: vesicular stomatitis virus (VSV, *Vesiculovirus* genus family *Rhabdoviridae*), bluetongue virus (BTV, *Orbivirus* genus, family *Reoviridae*), bovine viral diarrhea virus (BVDV, *Pestivirus* genus, family *Flaviviridae*) type 1 and 2, malignant catarrhal fever virus (MCFV, *Rhadinovirus* genus, family *Herpesviridae*), bovine herpesvirus-1 (BoHV-1, *Varicellovirus genus,* family *Herpesviridae*), parapox virus complex (PPV, *Poxviridae, Parapoxvirus*), and rinderpest Virus (RPV, *Morbillivirus* genus, family *Paramyxoviridae*).

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