RESEARCH REPORT
SWINE HEALTH

Title: PCR-on-a-Chip for the Identification and Control of Porcine Reproductive and Respiratory Syndrome Virus – NPB #04-198

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Abstract

We have been working on the development of a micro-fluidic DNA assay with the goal to quickly identify the introduction of PRRSv in pig herds in order to eliminate the transmission and spread of the virus. The project involved developing and integrating many structural components of the micro-fluidic DNA assay. Our work has led to the development of successfully operating micro PCR chambers, micropumps and capillary electrophoresis systems. A thermal cycler with flexible PCR cycle control was designed and implemented using Labview software. We have also completed fabricating and testing of gel electrophoresis micro-channels. Our results have shown that we can achieve ramp-up and -down heating times that are almost $1/10^{th}$ of any conventional system. Amplification has been tested using two different dilution ratios with extremely low (picogram level) template DNA concentration on this platform. We have developed a chamber design which demonstrates negligible non specific binding of the template DNA. We have been able to successfully demonstrate multiple usage of this chip without cross contamination from the previous run by introducing a wash step in between with elution buffer and RNase free water. This observation and the fluorescence studies provide us a basis of demonstrating the reusability of this platform for multiple PCR runs. The capillary electrophoresis system was fabricated using a 225 micron PDMS glass capillary filled with ethidium bromide doped agarose sample. Two thin platinum wires were used as electrodes and 300 V DC was supplied in the agarose capillary. A cross capillary was used to load a 100-1000bp DNA ladder and this was successfully electrophoresed inside the capillary in about 50 secs. Additionally, we amplified a 527-bp viral DNA of Infectious Bovine Rhinetracheotis (IBR) on a microchip scale because of its high gel fluorescence response of this stain (reported earlier). We have also amplified 750 bp PRRS isolate. In addition, we developed a novel gel material using doped Platinum nanoparticles in which a substantially enhanced mobility of the IBR and PRRS stains during electrophoresis was achieved.

Introduction

The ability to detect PRRS is imperative in order to reduce devastating production losses. While great strides have been made in detection of the virus, many technological problems remain. The current diagnostic techniques rely on serologic tests such as enzyme-linked immunosorbent assay (ELISA). Unfortunately, they suffer from multi-step sample preparation (filtering, chemical processing, cell lysing, washing, etc.), false positives, and poor ease-of-use. Additionally, high-sensitivity techniques, such as polymerase chain reaction (RT-PCR) are expensive and require time-consuming sample amplification. Such techniques are therefore incapable of rapid detection at production sites and must be run in a diagnostic laboratory. Our objective was to develop a unique micro-fluidic DNA assay or PCR-on-a-chip for field testing. The completed future device will be a disposable microfluidic cassette mounted on an in-house designed light proof casing containing a current controller box, an excitation source, and a detector setup. The cassette will be connected by silicone tubing to the inlet outlet fluidic ports fixed on the body of the casing for fluid exchange. The device will be a self-sufficient, portable on-chip DNA micro analyzer, which will perform field analysis with high speed and accurate detection of PRRS. We envision a network of multiple devices capable of remote data transmission and receiving in such applications. This vision necessitates a multidisciplinary research platform, which promises a successful integration of engineering with life sciences. The device is unique because of its higher potential of detection triggered by its capacity to analyze extremely small microliter size fluid volumes and also its simple, rugged fabrication using inexpensive materials.

Our device will have several advantages over conventional bench top systems which primarily include an overall reduction in size, reduced use of reagents, decreased power requirements, increased speed and accuracy of analysis, and increased portability for field use. Our device will speed up and simplify PRRSv detection by integrating a variety of functions such as sample preparation, DNA amplification, pumping, valving, and detection onto a single platform with minimal required human intervention.

Objectives

The goal of the project was to develop a portable PCR based DNA micro-analyzer. The design included a micro-fabricated silicon PDMS (polydimethyl siloxane) platform for amplification of target DNA, on-chip fluidic transport of the amplified sample into an agarose gel channel, and detection using an array of planar waveguides. The waveguides are placed parallel at a predetermined spatial separation according to the nature of the targets. (See Figure 1). An off-chip signal detector, connected through optical fibers to the waveguide set, amplifies and feeds the signal differential to a data acquisition (DAQ) system, which plots a temporal chart of the intensity. The research question that was to be answered was whether the first generation integrated PCR based DNA micro-analyzer will be able to achieve a fluidic transport at this microscopic length scale without compromising the sensitivity and specificity to detect field isolates. *The bigger question posed was whether the sensitivity and specificity can surpass standard PCR.* This project was broken down into discrete tasks with the following research objectives:

1) To integrate the individual components of the device onto a single chip design (micro-PCR chamber, temperature controller/heaters, peristaltic pumps, gel electrophoresis micro-channels, and solid core waveguides).

2) To achieve an all micro-scale fluid transport by using peristaltic action.

3) To develop gel electrophoresis micro-channels and interface with solid core waveguides to efficiently collect emitted fluorescence.

4) To test the sensitivity and specificity of the device using PRRS field isolates and compare results to standard PCR.

Five tasks were assigned in order to achieve our specific objectives:

Task 1: Fabricate on-chip PCR amplification chamber and heaters.

Task 2: Fabricate on-chip micro-scale fluid transports.

Task 3: Fabricate and test on-chip micro-channel gel electrophoresis.

Task 4: Fabricate and test on-chip solid core waveguides.

Task 5: Test the device to determine if (1) an all microscale fluid transport is achieved and (2) sensitivity and specificity of PRRS field isolates is achieved.

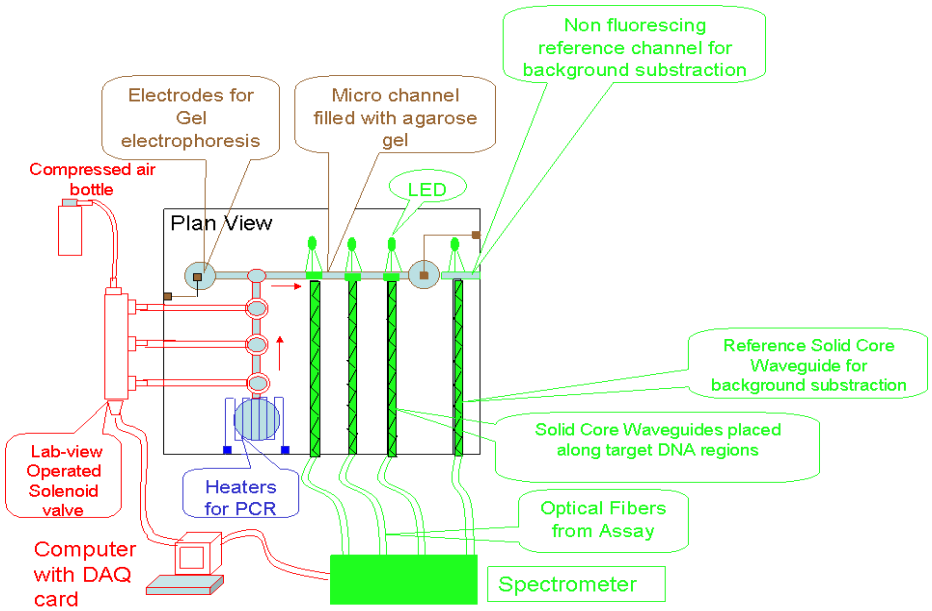

Figure 1. Schematic of the overall DNA assay units

V. and VI. Materials, Methods and Results

Because of the nature of this project, the Materials & Methods and Results will be co-listed. This arrangement will better elucidate the individual tasks and the results of each task.

Task 1. Fabricate on-chip PCR amplification chamber and heaters (color blue in Figure 1): Figure 2 shows the schematic of the PCR chamber and heaters. The device is a replica molded piece of PDMS with a chamber (diameter= 3mm, depth = 450 microns, volume = 3µl) and channels (depth = 450 microns, width = 1mm, length =12mm) leading to the inlet/outlet reservoirs (diameter = 1.5mm, depth = 450 microns, volume = 1.5µl). This was bonded to a 25.4mm X 25.4mm SOG (spin on glass) coated silicon wafer. The silicon has a 1 micron thick oxide layer on both sides which helps insulate the heater fins from each other. The inlet/outlet ports were mounted over the inlet/outlet reservoirs and used for guiding the PCR mix into the chamber. A thermocouple fitted snuggly inside a glass pipe with its sensing end projected outside the pipe and was coated with a thin layer of epoxy. It was inserted into a predrilled hole on the top of the PDMS chamber. The glass pipe was designed as an interference fit with the hole. A layer of epoxy was brush-coated around this housing for proper sealing. The thin layer of epoxy at the tip of the thermocouple protects the TAQ polymerase from direct contact with the tip.

Serpentine heater design: We also developed a serpentine heater design to obtain a fast heating/cooling rate, due to an overall lower thermal mass for our PDMS-silicon chips. The heaters were fabricated on a silicon wafer with a thermally grown oxide layer of 1 micron thickness using standardized sputtering and lift off proc

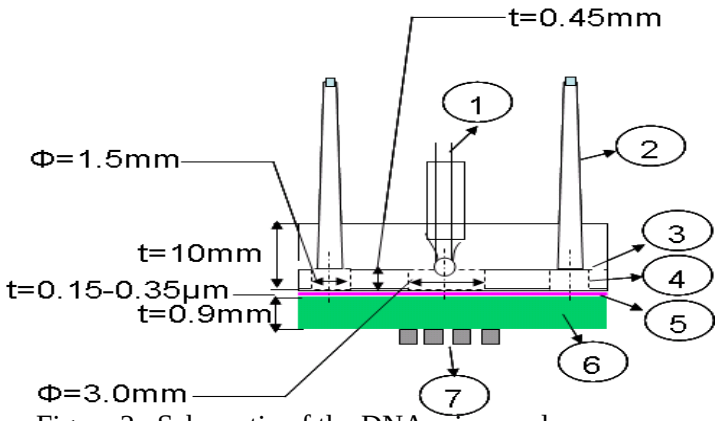

Figure 2. Schematic of the DNA microanalyzer.

	Glass housed thermocouple epoxied on the tip
2	Epoxied inlet/ outlet ports
3	Poly Dimethyl Siloxane (Channels)
4	Inlet outlet reservoirs
5	Spin On Glass layer
6	Thermally oxidized Silicon wafers
7	Heaters

esses. We used 20 nm 'Ti' and a 400 nm 'Pt' sputtered layer. The surface of the silicon wafer opposite to the heater structures was spin-coated with a layer of SOG using varied spin speeds from 1000 [film thickness= 1340nm] to 10,000 rpm [film thickness= 140nm] in order to produce different thicknesses. We measured the temperature difference and optimized SOG thicknesses across both the faces by placing two thermocouples over the heater side and the SOG coated side. The PDMS channels were fabricated by standardized SU8 lithography and PDMS casting processes using RTV 615. The inlet and outlet ports were mounted on the two reservoirs before casting the liquid PDMS. We used a K type, 5SRTC series, thermocouple for sensing the temperature of the chamber by inserting it into a glass pipe and snuggly fitting the tube in another predrilled hole in the PDMS over the chamber. Quick drying epoxy was used to provide better sealing. The wafers were irreversibly sealed in a plasma etcher using 20W RIE power, 900 mTorr chamber pressure, 182 sccm oxygen flow rate and 35 secs time of exposure. These parameters obtained were suited to a maximum bond strength condition.

Thermal cycling system: We have established an automated thermal cycling system control using National Instrument's Labview software. The control is executed by a current controller with a power MOSFET serving as a PWM (Pulse Width Modulation) device by varying the duty cycle of the gate voltage as a switch.

Results of polymerase chain reaction using the chip: We used Infectious Bovine Rhinotracheitis (IBR) virus as the test assay for our on chip studies. We used this test assay for testing on chip amplification due to two main reasons. One, these are a long thermal cycling times which evaluate the reliability of our device and two, a strong fluorescence response of the viral genome in any standard laboratory gel setup is obtained. In the assay used for our studies, a 527 base pair product was amplified using a 51 cycle PCR process.

We performed trials with 7 ng of template DNA using our on-chip device and got conformity of amplification when the PCR product was electrophoresced with a standardized gel slab made out of a 2% agarose solution. Each time a portion of the sample was amplified in the standard PCR machine for conformity of amplification. A 5 µl sample volume was injected through the inlet port in order to fill the chamber and the channel. This was firmly capped on both the ports and the sample was then amplified in the on-chip chamber. Then the amplified sample was removed from the micro-chamber using a hypodermic syringe. The extracted sample was run in four side-by-side wells of the gel. A 180 V DC potential was applied across the gel slab for 30 mins for electrophoresis. The post electrophoresis gel image for both the conventional system and our microchamber indicated bands at the 527 bp region; thus demonstrating amplification.

1 2 3 4

Repeated amplification of the PCR mix in our microchamber shows that the hydrophopically recovered SOG and PDMS surfaces are *not* PCR inhibitors. (Silicon or glass substrates which have been widely used in earlier PCR on chip systems are inhibitors.) One prominent cause of contact inhibition of the PCR reaction is the ability of the DNA to preferentially bind to some substrates. This is primarily due to DNA being negatively charged will bind to polar groups on any surface. Both our PDMS and SOG have shown post exposure surface relaxation. They exhibited a gradual methylation and dehydroxylation of the surface at increasing post exposure times making the surfaces more non polar. From the repetitive amplification results of the on-chip device, we saw that the SOG and PDMS forming the inner walls of the chamber and ports provide a *completely inert* surface with no DNA binding.

We have also tested the sensitivity of our chamber by diluting the template DNA with RNase-free water. Results have been obtained with both a conventional thermal cycler and the on chip amplifier. We started with a standard DNA sample (conc.= 7 ng/µl), as used in the conventional setup, and diluted this with RNase water in the ratios 10:1, 100:1, 1000:1, 10000:1 and 100000 :1. We took 5 microliter volumes of each of these dilutions and successfully amplified it in the conventional thermo-cycler. Additionally, we could successfully obtain stains up to 100000:1 dilution level (corresponding to .07picogram/µl) after gel electrophoresis of PCR products as amplified in a conventional machine. Figure 3 shows a post electrophoresis gel image of this trial. Well no.1 was used to load the DNA ladder, No.2 for sample from the conventional setup, No. 3 for the on chip chamber sample and No. 4 for the sample from one of the ports. The image

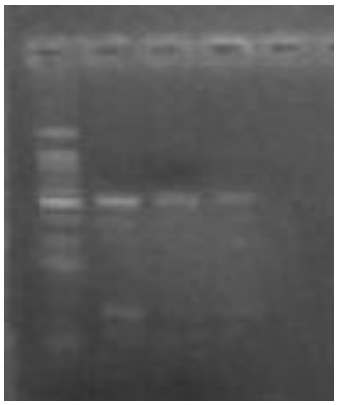

Figure 3: Slab gel image of a 100000:1 dilution of initial template by amplifying on the on-chip device.

shows bands in all the tracks which prove that our chip can amplify at highly diluted concentrations of the initial template.

In summary, the process begins by injection of a predetermined volume of PCR mix (~4-5 microliters) with a fixed volume of sample DNA inside a micro chamber molded in PDMS. A small current controller operated by a computer code feeds a set of microfabricated platinum heaters placed below the PCR chamber. Another microfabricated, well characterized platinum film is used beside the heater as an RTD (resistance based temperature detector) for monitoring and controlling the response of the current controller. A Labview code is used to operate time and temperature control. As the sample is injected and floods the reservoir, the chamber acts as a valve thus restricting all PCR mix to reservoir. The cap remains closed during the heating cycle to prevent evaporation effects. Our studies also indicated that this evaporation rate can be further reduced by capping the PCR mix with mineral oil.

Task 2. Fabricate on-chip micro-scale fluid transport (color coded red in Figure 1): As shown in Figure 1, after completion of the PCR reaction, a valve is opened and the products are transported into the reservoir. A peristaltic micropump system has been developed that maneuvers the fluid using a discreet traveling contractile effect on chip.

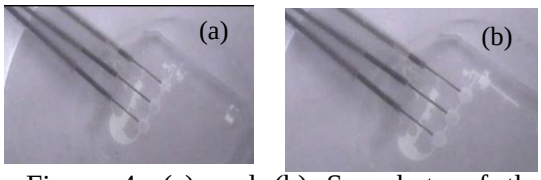

Figure 4: (a) and (b) Spanshots of the fluorescence dye flowing through the micro-channel taken at 4 secs. interval.

The pump system utilized a discrete peristaltic action achieved by creating a traveling contractile effect. A traveling pinching action was provided by compressed nitrogen controlled by a set of Labview controlled solenoid valves. The primary advantages of such fluid handling mechanisms are bidirectionality of flow and a high fluid transport rate (9~12 microliters/min.). We plan to use this micron scale fluid transport to move samples inside the microchip. This results in minimal human intervention and a high reusability. [Fig. 4(a) and (b)] Peristalsis is a motion of fluid through a channel by means of a traveling contractile across it. The direction of motion of the fluid is same as the contractile.

Task 3: Fabricate and test on-chip micro-channel gel electrophoresis (color coded brown in Figure 1):
We have successfully amplified and electrophoresed a 750 bp PRRS isolate and observed an enhancement in its mobility with addition of platinum nanoparticles. The mobility of the DNA stains was calculated using the one dimensional mobility equation [1].

$$\mu = v / E \qquad (1)$$

where , μ = mobility of the stain, v= Velocity (cm/ sec.), E= Electric Field (V/cm). The mobility values are calculated from the various images and these are plotted with the voltage values [Fig. 5]. We have observed an increase in the mobility of the DNA stain at lower electric fields (8 V/cm) from 4.95E-5 cm^2/ V.sec to 9.11E-5 cm^2/ V.sec(1.8 times). No substantial difference was observed in mobility values below this electric field.

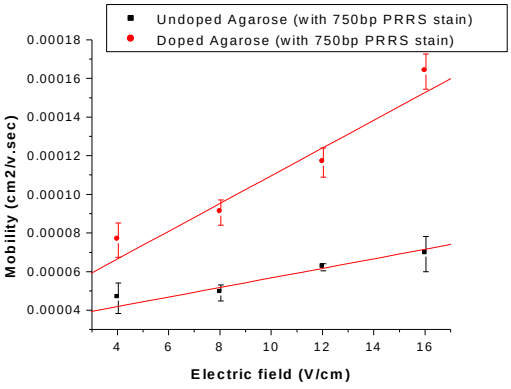

Figure 5: Mobility plots for plain and doped agarose.

We have observed a 1.8 fold increase in the DNA mobility at 8 V/cm electric field and a *five* fold enhancement in the conductivity due to the inclusion of the platinum nano-particles in the gel matrix. At electric fields below this, the mobility of different DNA strands do not show any remarkable difference in both doped and plain agarose gels. The difference in mobility increased at higher field values as the field was increased to 16 V/cm. We hypothesized that the enhanced DNA mobility arises from an enhancement in the dielectric constant due to presence of the dispersant phase in the agarose medium. The slope of the mobility versus electric field curve doubles in case of the doped agarose showing possibility of capillary electrophoresis at lower

electric field values. We have demonstrated capillary electrophoresis in plain agarose capillaries in an earlier work by using field values in the range of 80-100V/cm (applied voltage =300 V). We have successfully electrophoresed a 100-1000 bp gene marker by using this gel material with a high resolution indicating no association of the fixed platinum phase with the sample. A set-up of this system is shown in Figure 6. [A paper has been submitted to Analytical Chemistry].

Trial 1& 2 with PRRS isolate (750 bp):

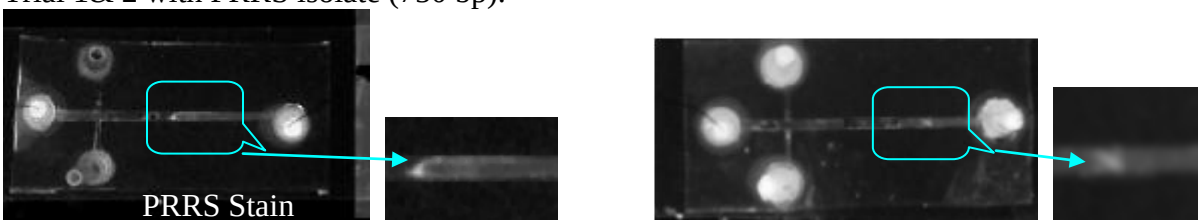

Fig. 6(a) Trial 1: Capillary electrophoresis on a chip (b)Trial 2: Capillary electrophoresis on a chip.

Task 4: Fabricate and test on-chip solid core waveguides for fluorescence collection (color coded green in Figure 1): We have been working on developing PDMS cladding, SU8 core lightguides. However, SU8 being a thermally unstable material did not provide a reliable responses during testing. Thus, we modified our initial design of utilizing SU8, and tried synthesizing a high refractive index PDMS composite by mixing PDMS with high band gap nanoparticles like titanium dioxide, zinc oxide etc. This material would be injected into the core of PDMS channels and thus obtain the requisite refractive index difference to obtain lossless waveguiding. These on-chip waveguides placed in a transverse direction to the microfabricated capillaries will capture the fluorescence signals from the electrophoresced DNA stains and then guide them to an off-chip spectrophotometer. Unfortunately, these designs have thus far failed to produce the desired low loss waveguiding capabilities.

Task 5: Testing of the device: As noted above, we have performed high quality studies on the previous 4 tasks, which have resulted in the development of the components for PCR amplification, microfluidic transport, gel electrophoresis and optical detection with on-chip waveguides. We have completed our final task by amplifying and electrophoresing PRRS field isolates. We have successfully demonstrated amplification and capillary electrophoresis using PRRS isolate and also a 100-1000 bp gene marker. We have been able to successfully reduce the electrophoresis time by a factor of 40 from standard gel electrophoresis. We have also developed a novel doped gel matrix which enhances stain mobility of nucleic acids (Tested using a 750 bp PRRS isolate). This new material is developed by doping agarose gels with Platinum nano-particles. This material can be utilized in micro-channels to perform low voltage capillary electrophoresis. Details on our PCR on a chip design can be found in one of our submitted publications [Bhattacharay, S. V. Korampally, Y. Gao, S.A. Grant, S. Kleiboeker, K. Gangopadhyay, S. Gangopadhyay. A novel on-chip platform for amplification of DNA. In print by *JMEMS*, 2006.]

Discussion

We have made much progress and many of our goals were accomplished in the grant period. We have also produced publications, abstracts, and presentations at national conferences. We have optimized the micro-fabrication and design process of an on-chip DNA amplifier for amplification of a 527 fragment of the IBR virus genome as well as a 750 bp PRRS stain using a 51 cycle PCR process and have successfully amplified 0.07 picograms/µl of template using this device. Fluorescence studies performed on the chip with labeled DNA indicated no non-specific binding of the DNA to the hydrophobic walls of the chamber. The chip is extremely durable and can be thermally cycled for a significant amount of time without any damaging thermal stresses, which is a main reason for failure of glass PDMS micro-chambers.

We have further developed a serpentine heater design. There was a considerable increase in the ramp up and down rates with the serpentine design over its continuous counterparts. In comparison to identical architectures, we have been able to achieve similar ramp up and down rates (3 deg. C/sec) at lower input power values (4.77 W). We have also developed a new regime of PDMS to SOG bonding. We performed contact angle bond strength studies for SOG-PDMS bonding, leading to the development of a set of exposure parameters for maximum bond strength. This maximum bond strength was 83 psi, using the standardized blister test. This was much higher in comparison to PDMS-thermally grown silicon dioxide (only 10 psi). We characterized the silicon dioxide surface immediately after plasma exposure with an ATR-FTIR, which indicated small numbers of Si-OH in comparison to another exposed SOG surface. The differential in the surface hydroxyl groups are a possible reason of lower bond strengths of the PDMS-SiO_2 bonds. We have tested our chip with sequential positive and negative control and observed no bound DNA left over from previous amplification runs after the wash step. We also reduced the ramping up and down time for the 51 cycle process to an order of magnitude less than that required in a conventional setup.

We have been able to successfully electrophorese a 750 bp PRRS stain in a microcapillary 225 microns deep. We have also electrophoresed a 100-1000 bp DNA ladder in another trial. The ladder moves as a plug for about 45 secs before it separates into different stains. The mobilities of the 750 bp PRRS stain are calculated in the capillary as 7.8E-4 and 7.5E-4 cm2/ V.sec respectively in two consecutive trials whereas for the ladder it is calculated to be 9.1E-4 cm2/Vsec. We synthesized a new gel material by doping agarose with Platinum nanoparticles, which enhanced sample mobility and increased conductivity. The sample mobility in the composite increases from 4.95E-5 cm^2/ V.sec to 9.11E-5 cm^2/ V.sec(1.8 times) at low (8V/cm) field values. The slope of the mobility versus electric field characteristics increase by a factor of 2. The conductivity of the new composite is found to increase five folds. We believe this mobility increase comes from an enhancement of dielectric constant of the medium. The overall objective to perform capillary electrophoresis at field applicable low voltages was achieved.

Lay Interpretation

We have been working on the development of a micro-fluidic DNA assay with the goal to quickly identify the introduction of PRRSv in pig herds in order to eliminate the transmission and spread of the virus. We have successfully designed and fabricated the components for DNA amplification, microfluidic transport, gel electrophoresis and optical detection with on-chip waveguides. Research into more durable waveguides is needed in order to fabricate a field unit. The realization of all the individual modules is an important step towards the complete fabrication and integration of an inexpensive DNA microanalysis platform for fast and accurate identification of target DNA molecules that will be amendable for field applications. Please contact Sheila Grant, Dept. of Biological Engineering, University of Missouri-Columbia (grantsa@missouri.edu) for further information.