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EXP-SA: Explosives Tracking: A Microsystem for Detection of Bacterial Endospores as Self-Replicating Nucleic Acid Taggants

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Title:
EXP-SA: Explosives Tracking: A Microsystem for Detection of Bacterial Endospores as Self-Replicating Nucleic Acid Taggants

Project Participants

Senior Personnel

Name: Millard, Paul
Worked for more than 160 Hours: Yes
Contribution to Project:

Name: Pereira da Cunha, Mauricio
Worked for more than 160 Hours: Yes
Contribution to Project:

Name: Singer, John
Worked for more than 160 Hours: Yes
Contribution to Project:

Post-doc

Graduate Student

Name: Peimer, Joshua
Worked for more than 160 Hours: Yes
Contribution to Project:
Joshua is involved in the genetic engineering and detection aspects of the project. He started in the group in August 2008 and is now making major contributions to the project.

Name: Harris, Megan
Worked for more than 160 Hours: Yes
Contribution to Project:
involved in development of multiplex on-chip PCR analysis and genetic engineering of Geobacillus

Name: Lyford, Timothy
Worked for more than 160 Hours: Yes
Contribution to Project:
involved in development of surface acoustic wave based fluid transport and microbial lysis

Undergraduate Student

Name: Amatya, Jyoti
Worked for more than 160 Hours: No
Contribution to Project:
Ms Amatya is involved in the design, fabrication and testing of an on-chip bacterial culture chamber.

Name: McPhee, Shawn
Worked for more than 160 Hours: No

Contribution to Project:
Carried out work with endospore germination and completed his senior capstone research in biological engineering using this system.

Name: McCarthy, Owen
Worked for more than 160 Hours: No
Contribution to Project:
Owen completed a senior capstone project involvine heat transfer in a PDMS/glass microfluidic device. THe project involved fabrication, modeling, and testing of a multiplex PCR device.

Name: Wagner, Chelsea
Worked for more than 160 Hours: No
Contribution to Project:
Chelsea was integrally involved in construction of PDMS microdevices and in finding methods to deposit and rehydrate solutions in microchannels.

Name: Dullinger, Michelle
Worked for more than 160 Hours: Yes
Contribution to Project:
Capstone project - molecular biol of Geobacillus

Name: Quintillani, Alexis
Worked for more than 160 Hours: No
Contribution to Project:
genetic engineering of G. thermoglucosidasius

Technician, Programmer

Other Participant

Name: Cariou, Alois
Worked for more than 160 Hours: No
Contribution to Project:
Visiting student form I.U.T. of Quimper - working on the molecular biology of Geobacillus

Research Experience for Undergraduates

Name: Porter, Laura
Worked for more than 160 Hours: Yes
Contribution to Project:
Laura performed many of the critical experiments in the genetic engineering aspect of the project. She is continuing on in this role for at least the next 6 months.

Name: Patel, Nimesh
Worked for more than 160 Hours: Yes
Contribution to Project:
Nimesh worked on the DNA extraction, amplification and detection subsystems.

Name: Ortiz, Alexander
Worked for more than 160 Hours: Yes
Contribution to Project:
Alex was involved in the development of an indium tin oxide glass substrate heating for microfluidic devices. He focused on temperature control, ramp rate and device uniformity. He will likely continue his work in the from of a senior capstone project in biological engineering.

Name: Gilbert, Erin

Worked for more than 160 Hours: Yes

Contribution to Project:

Research and develop[ment of on-chip multiplex PCR

Name: McBrayer, Kepra

Worked for more than 160 Hours: No

Contribution to Project:

worked on improvement of SAW-based microbial lysis platform

Organizational Partners

Other Collaborators or Contacts

None

Activities and Findings

Research and Education Activities: (See PDF version submitted by PI at the end of the report)

Findings: (See PDF version submitted by PI at the end of the report)

Training and Development:

As stated in the Activities and Findings section of this report, during the year that this project has been active, it has provided direct training for three undergraduate students and two graduate students. Teaching materials originating from this work have been incorporated into introductory engineering workshop designed to help high school students to understand biological engineering. Methods described ere have been adopted for use in undergraduatre laboratories directed toward sensors and microfluidic devices. In addition, discussion of project goals and technical details with NSF-RET participants at UMaine has helped to extend their range of research experience in both the physical and biological sciences.

One of the most important contributions of the current project to the University of Maine, and to the research community, has been the cross-training of students in biology and engineering brought about through the multidisciplinary nature of the work. Even at this early stage, the enhanced interaction between our microbiology/molecular biology collaborators and engineers has led to the development of new projects and new solutions to ongoing research. We have also found that the techniques that are being devised translate very well into educational tools, both for the PI's present courses at the university, and for regularly offered programs designed to introduce high school students to biological engineering and research.

Outreach Activities:

It is expected that in addition to the technology for taggant generation and recognition that is the focus of the project, the research will ultimately generate methods and devices for microbial detection and identification that are faster and less expensive than existing technologies. This outcome will permit a broad range of outreach activities. THe PI has communicated some of the findings of this work and is seeking to apply some of the strategies to human diagnostics.

Journal Publications

Books or Other One-time Publications

Web/Internet Site

Other Specific Products

Product Type:

Conference Proceeding - web search tool not available

Product Description:

IEEE International Ultrasonics Symposium
The work was presented at the 2012 IEEE International Ultrasonics Symposium held in Dresden, Germany. In addition to a publication in the conference proceedings, the paper, entitled ?Cell Lysis using Surface Acoustic Waves for Sensor Applications,? was presented as an oral presentation and received 2nd place in the student paper competition.

Sharing Information:

The work will be in the published proceedings of the IEEE

Contributions

Contributions within Discipline:

This is an integrated research and educational project directed toward the production, detection, and identification of bacterial endospore taggants for explosive tracking. A powerful strategy for tracking and identifying specific lots of explosives is the incorporation of identification tags, or taggants. We are combining the bioengineering of Geobacillus stearothermophilus endospores with microdevices for sample processing and taggant identification. The project involves the generation of Geobacillus endospores with unique genomic DNA sequences. Further, surface acoustic wave (SAW) devices are being designed and fabricated to germinate spores, lyse vegetative bacteria, transport, mix, and heat samples. Subsystems are in development for DNA isolation, and fluorescence-based DNA identification. While the most immediate application of the research is related to stemming terrorist activities, the anticipated fundamental advances in bioengineering and sensor science and engineering will have significant societal relevance to other applications, including first-responder activities, healthcare, food safety, and pollution avoidance and mitigation. In addition to the technical objectives, this program is providing a multidisciplinary learning experience to students ranging from high school to graduate student level in the area of bioengineering.

Although it is early in the life of the project, we have made the most significant progress in two fundamental areas: (1) We have perfected methods for transforming Geobacillus bacteria with foreign DNA rapidly and with high efficiency, which is essential if endospore taggants are to be generated quickly and accurately. (2) We have improved on current methods for microbial detection via DNA identification by making progress in the creation of a highly sensitive, versatile, and inexpensive method for direct DNA identification from bacteria by PCR amplification and subsequent DNA hybridization. Ongoing work is directed toward the design and fabrication of SAW devices for sample processing operations.

Contributions to Other Disciplines:

This is an inherently multidisciplinary project that involves a range of science and engineering disciplines. At this stage, the overall project has not generated significant contributions to disciplines of science or engineering outside of those originally identified. Subprojects encompassed by the primary project have begun to yield findings that should ultiumately be applicable to both environmental monitoring and medical testing and diagnosis. in particular, SAW-based bacterial lysis technology is applicable to other cell types, including blood cells. Multiplex on-chip PCR methods will have both environmental monitoring and medical applications.

Contributions to Human Resource Development:

As stated in the Activities and Findings section of this report, during the second year that this project has been active, it has provided direct training for several undergraduate students and three graduate students. At least two new undergraduate students will begin work on the project in Fall 2010. New teaching materials originating from this work have been incorporated into introductory engineering workshops designed to help high school students to understand biological engineering. In addition, discussion of project goals and technical details with NSF-RET participants at UMaine has helped to extend their range of research experience in both the physical and biological sciences.

Contributions to Resources for Research and Education:

One of the most important contributions of the current project to the University of Maine, and to the research community, has been the cross-training of students in biology and engineering brought about through the multidisciplinary nature of the work. Even at this early stage, the enhanced interaction between our microbiology/molecular biology collaborators and engineers has led to the development of new projects and new solutions to ongoing research. We have also found that the techniques that are being devised translate very well into educational tools, both for the PI's present courses at the university, and for regularly offered programs designed to introduce high school students to biological engineering and research.

Contributions Beyond Science and Engineering:

It is expected that in addition to the technology for taggant generation and recognition that is the focus of the project, the research will ultimately generate methods and devices for microbial detection and identification that are faster and less expensive than existing technologies. Our SAW-based lysis technology has been developed partially through the involvement of undergraduate students, in collaboration with a well established diagnostic company in Massachusetts.

Conference Proceedings

Categories for which nothing is reported:

Organizational Partners

Any Journal

Any Book

Any Web/Internet Site

Any Conference

Introduction

A no-cost extension for this project was requested during the latter part of Year 3 in order to allow completion of the project goals. Progress made during Year 4 of the project involved three linked areas of research. Each part is described in detail below.

Part I. Genetic Engineering of *Geobacillus* endospores

Focus. The genetic engineering of *Geobacillus thermoglucosidasius* required to generate transformed bacteria with stable unique DNA sequences to be used as taggants.

Optimization of transformation. During the end of Year 3 and the first half of Year 4 we optimized electroporation as the most cost-effective, time-effective means of transformation of *Geobacillus thermoglucosidasius* NUB3621 with plasmid DNA.

Optimal electroporation conditions. Optimal conditions for electroporation of frozen cell suspensions included 60 µl of cell suspension in a 1 mm gap chilled cuvette. Optimal pulse conditions were 20 kV/cm, a resistance of 400 ohms, and a capacitance setting of 25 µF. Time constants were in the range of 4–5 msec and 25–50 transformants/µg of DNA were obtained. Cell survival was 10.2–10.6%.

Other selected findings include:

1. *Geobacillus thermoglucosidasius* NUB3621 can be transformed with CsCl-purified plasmid DNA isolated from *E. coli* strains and from NUB3621. However, DNA isolated from NUB3621 transforms with 50–100-fold greater efficiency, suggesting that NUB3621 is likely not Hsr^- as was described in the literature.
2. NUB3621 can be transformed with alkaline lysate-isolated plasmid DNA only from NUB3621.
3. The DNA threshold for isolating transformants was high at about 25 µg. No transformants were ever obtained in reactions containing 0.2-, 0.5-, 1-, or 5 µg of plasmid DNA.

Preparation of electrocompetent cells. A 25 ml 2TY medium starter culture was grown overnight at 52°C. The culture was diluted 1:10 in 50 ml of prewarmed 2TY medium and grown at 52°C for 4.5 h until the A_{600} of the culture reached 3.6–4.0. Cells were chilled, harvested by centrifugation, and were washed sequentially with 30-, 20-, 10-, and 4 ml of ice-cold electroporation medium (EM). The final cell pellet was resuspended in 2 ml of ice-cold electroporation medium and 130-µl aliquots of electrocompetent cells were quick-frozen and stored at –85°C. Cells were thawed on ice and 60-µl aliquots were added to 1 mm gap chilled cuvettes for electroporation.

Electroporation. DNA was preincubated with 60 µl of electrocompetent cells on ice for 10 min prior to pulsing. After the pulse, cuvettes were immediately returned to ice. Cells were recovered for expression by rinsing cuvettes twice with 1 ml of prewarmed 50°C 2TYG medium. Expression was carried out at 50°C with gentle shaking (150 rpm) for 1–8 h. Cells were harvested and plated on mLB plates containing chloramphenicol (7 µg/ml) to select transformants. Maximum numbers of transformants were repeatedly obtained after 4 h of expression. Numbers of transformants obtained at 5–8 h of expression declined from the peak observed at 4 h.

DNA preparation–EDTA effects. When increasing amounts of transforming DNA in TE were used for electroporation. A sharp drop in transformation was observed at 300 µg of DNA/ml when contributing EDTA concentrations exceeded 200 µM. Precipitating and resuspending the DNA in 0.1X TE relieved the inhibition and resulted in a linear increase in transformants beyond 400 µg of DNA/ml. Studies using a single DNA concentration in 0.1X TE and progressively increasing concentrations of EDTA in the electroporation mix revealed that low concentrations of EDTA (150–200 µM) stimulate recovery of transformants 2.5–3 fold, while concentrations of EDTA much beyond 250 µM result in declining recovery of transformants. Similar studies with varying Tris concentrations resulted in maximum numbers of transformants when Tris was present at 1.4 mM in the electroporation mix. The suggestion that EDTA might be protecting transforming DNA from potential nuclease activity was tested by the inclusion of a 2-, 5-, and 10-fold excess of non-transforming genomic DNA from *Vibrio anguillarum*. No stimulatory effect on transformation was observed by the inclusion of heterologous DNA.

Our optimized conditions for electroporation at present include using plasmid DNA dissolved in 0.1X TE with the addition of Tris-Cl (pH 8.0) and EDTA (pH 8.0) to all electroporation reactions at 1.4 mM and 200 µM, respectively.

Plasmid stability studies. In order to be used as a taggant, the selected DNA sequence must be stably maintained in the absence of selection during both sporulation and during germination/outgrowth. pNW33N and pNW33N-kan were used for stability studies. pNW33N-kan carries a cloned 1.5 kb kanamycin resistance determinant that is silent in NUB3621 and is expressed (and detectable phenotypically) only in Gram-negatives.

Retention of pNW33N during sporulation and germination. NUB3621(pNW33N) was grown in 50 ml of mLB Cm7 at 50°C for 96 h until the culture cleared and had undergone sporulation. Spores were harvested from 40 ml of culture, resuspended in 400 µl of mLB, and were heated at 95°C for 10 min to kill any remaining vegetative cells. Spore suspensions were then incubated for 2 h at 50°C to allow germination to begin and to permit the expression of chloramphenicol resistance. Germinating spores were then serially diluted and spread onto mLA plates and onto plates of mLA Cm7. An average of 85 colonies grew on triplicate mLA plates and 87 colonies on triplicate mLA Cm7 plates. These results suggest that 100% of the vegetative cells that underwent sporulation included at least one copy of pNW33N within the spore. Repeated studies with pNW33N and pNW33N-kan resulted in 89–96% plasmid incorporation into spores.

To test for retention of pNW33N/pNW33N-kan during germination and outgrowth, several hundred colonies arising on mLA plates from the above experiments were picked to mLA Cm7 plates. In three replicates 89-, 84-, and 94% of spores that germinated and formed colonies on mLA plates were also chloramphenicol resistant and had retained pNW33n/pNW33N-kan.

Attached Materials.

Media.

mLB medium contained 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 3.125 ml of 1 N NaOH per liter and 1 ml of 0.59 M $MgSO_4$ $7H_2O$, 0.91 M $CaCl_2$ $2H_2O$, 0.04 M $FeSO_4$ $7H_2O$, and 1.05 M nitrilotriacetic acid (pH 6.6–6.8) per liter.

2TY medium contained: 1.6% tryptone, 1% yeast extract, 0.5% NaCl, 3.125 ml of 1 N NaOH per liter, and 1 ml of 0.59 M $MgSO_4$ $7H_2O$, 0.91 M $CaCl_2$ $2H_2O$, 0.04 M $FeSO_4$ $7H_2O$, and 1.05 M nitrilotriacetic acid (pH 6.6–6.8) per liter.

2TYG medium contained 2TY medium with 0.5% glucose.

EM–Electroporation Medium contained 0.5 M sorbitol, 0.5 M mannitol, and 10% glycerol.

TE 10 mM Tris-Cl–1 mM EDTA, pH 8.0.

Part II. Microsystem for Multiplexed DNA Analysis

Dried primers and Zen probe are not degraded by 80°C heating or exposure to coronal discharge. By studying the physical limits of the primer/probes appropriate choices could be made regarding the conditions used for device fabrication/assembly. Heat and coronal discharge treatments are potentially destructive to PCR primers and probes in PDMS devices.

PCR primers designed for pNW33N-kan5 for Km gene amplified in pNW33N-kan5 and genomic DNA but did not release the ADK Zen (i.e. provide a fluorescence negative control.) Dr. Singer's work showed that the pNW33N plasmid could be transformed into *G.t.* This plasmid has the ability to be transformed into both *G.t.* and *E.coli* allowing screening of ligations. The plasmid can then be extracted and transformed into *G.t*. The ligation of a kanamycin (Kan) gene block into pNW33N produced a gene block within the plasmid that could be used to ligate the synthetic DNA target sequence into that could then be screened for Kan sensitivity in *E.coli*. PCR of the *G.t.* genome with pKan5 primers and Zen ADK probe generated amplicons, however, this amplification did not occur in the target area of the Zen probe, leaving the fluorophore quenched. This provided a suitable negative control and illustrated a potentially beneficial feature, i.e. multiple probe sequences and target regions could be generated while maintaining only a few primer sequences.

Soluble bovine serum albumin (BSA) is concentrated as PDMS devices are re-filled to overcome vapor loss, resulting in inhibition of PCR. BSA is required in PCR reactions in the presence of PDMS or glass to prevent DNA absorption and to add stability to the polymerase. In excess, BSA inhibits PCR. During the initial 95°C heating step, water vapor was generated and lost into the PDMS. In order to overcome this loss, additional fluid containing 1mg/ml BSA was added, resulting in concentration of BSA over time. Alterations to the refilling protocol were adopted to prevent this concentration effect, using a dilute BSA solutions or water alone for refilling only with water. Neither modification of the protocol resulted in positive PCR reactions in the PDMS device.

Parylene coating of PDMS prevents water vapor loss. In order to prevent water vapor loss from PDMS devices, parylene coating of the PDMS channels was investigated. Parylene is a common fabrication material used for its biologically inert properties and vapor phase deposition. Parylene D was chosen for deposition because of its low water vapor permeability, but similar gas (air) permeability to PDMS. Maintaining gas permeability was important to permit filling of the devices under pressure in order to fill blind-ended channels with liquid. The dimensions of the PDMS device prevented complete vapor-phase parylene deposition, when devices were bonded to glass. Additionally, this method was not be suitable for this particular

device because primer/probe combinations must be deposited into the channels before the device was sealed to glass. While parylene could be deposited before glass bonding, this deposition interferes with PDMS bonding to glass. An optically clear adhesive film was obtained to potentially replace glass as the foundation for parylene coated PDMS channels, however, this film neither had the same rigidity as glass nor the ability to remain adherent when the channels were filled under pressure. Issues with the channel design and the PDMS substrate necessitated investigation into different device designs. New device design constraints included the incorporation of inlet and outlet ports, a bio-compatible substrate, and prevention of liquid loss during thermal cycling. A gasket-like structure was fabricated and tested with PDMS wells by applying force from above and thermal cycling. With the addition the optically clear sealing film no leakage was detectable, but a small amount of water vapor was still lost to the PDMS. A 4µm thick layer of parylene D was deposited onto the PDMS and, with the addition of the downward force and sealing film, no leaking or water vapor diffusion from the device was observed.

The next device design incorporated arrays of square microwells having well sides ranging from 3mm to 100µm, and connected by a single channel. Since target DNA flowed over multiple sample wells, a method was needed to prevent loss or primers and probe, as well as contamination between wells. The first method that was pursued was the encapsulation of preloaded primer/probe samples in agarose.

PCR can be carried out in 10µl PDMS microwells with Parylene coating if primers and probe are dissolved in 3% low-melt agarose (0.84% final concentration.) Low-melt agarose was used because it becomes a liquid at temperatures well below the maxima in the PCR thermal cycling profile. Encapsulation of primer/probe in agarose involved making a concentrated solution of agarose and subsequently adding primer/probe dilutions to the liquified agarose. Samples of liquified agarose were then refrigerated and allowed to harden. Once the agarose had set, a solution containing PCR master mix and target DNA was added.

This experiment was first preformed in 35µl volumes in 200 µl PCR tubes in a commercial thermocycler to determine whether the agarose was inhibitory to PCR. The volume of agarose added to each tube was equal to that of the primer, probe and water needed for a PCR reaction mixture without agarose. Agarose did not inhibit PCR to a large extent and the optimum initial agarose concentration was 3% (0.84% final concentration.) 3% (0.84%) agarose was used to test PCR in PDMS/parylene devices with well volumes of 10, 7, and 1.5µl. All devices produced positive results.

Unique primers for plasmid and genomic DNA designed to show unique sequence detection did not support PCR with vector DNA. The synthetic DNA sequence to be ligated into pNW33N must be unique in reference to the *G.t.* genome and pNW33N plasmid to prevent unwanted amplification during PCR. A target sequence was generated and screened against the known sequence of pNW33N. A complete genome sequence is not known for *G.t.*, and so the synthetic sequence was also screened against a catalogue of *Bacillus* species. A set of "GtTAG" primers and Zen probe were then designed for this DNA sequence. When tested in PCR with *G.t.* genomic DNA and pNW33N-kan5 plasmid DNA amplicons were generated but no fluorescence amplification was measured.

The total synthetic DNA sequence to be ligated into pNW33N-kan5 is currently being constructed using a 'building block' approach in order to reduce cost. This approach involved the combination of short oligos with shared sequences and other oligos having the desired

target sequence recognized by specific PCR primers. When ligated together in the appropriate orientation, these segments of DNA could then be incorporated into plasmids. This method of stepwise construction eliminated the need to have very long DNA sequences synthesized and purified, contributing design flexibility and greatly reducing the cost of each new target organism.

Design and fabrication of microwell arrays in silicon wafers. While the PDMS/parylene well-based design showed success at preventing liquid loss it did not meet the design requirement for inlet and outlet ports. The next generation device will incorporate wells connected by a single channel. The biologically inert material chosen for the next generation device is silicon. LASST personnel have vast experience with fabrication techniques using silicon.

Currently work involves developing protocols for the fabrication of a micro device using silicon and dry etch techniques. This work includes fabrication of an optically clear lid to seal devices, development of protocols for handling and dispensing hydrogels to individual wells, choice of effective microfluidic inlet and outlet ports, and development of syringe pump profiles for device filling.

Part III. SH-SAW Platform for Microdroplet Manipulation

Surface acoustic wave (SAW) devices were oriented to form an array surrounding a transport surface (see Figure 1). The array allows droplet directional control horizontally and vertically along the transport surface. This level of control will allow movement of multiple droplets at once and allow them to converge at a single point. Activating two interdigitated transducers (IDTs) that are facing one another results in mixing the droplet.

To properly operate all IDTs using one power source, the SAW devices were designed to operate at a single frequency; however, the wave propagation velocity of the substrate upon which the devices are fabricated, 128° Y-cut lithium niobate, is different in the vertical orientation(X-orientation), compared to the horizontal orientation (X+90° orientation; see Figure 2). To achieve a single operating frequency, the X+90° device's wavelength was decreased.

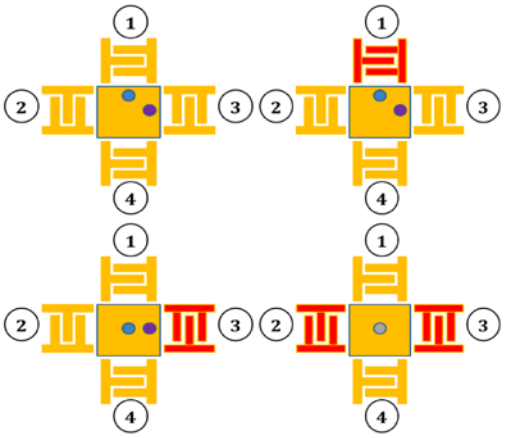

Figure 1. Two droplets on a transport surface are enclosed by four IDTs. When activated (shown in red), the IDT moves the droplet. Activating two IDTs results in droplet mixing.

The devices were fabricated with an expected operating frequency of 100 MHz due to the attainability of components at this frequency; however, measurements determined that the devices best operated at ~96.5 MHz (see Figure 3.)

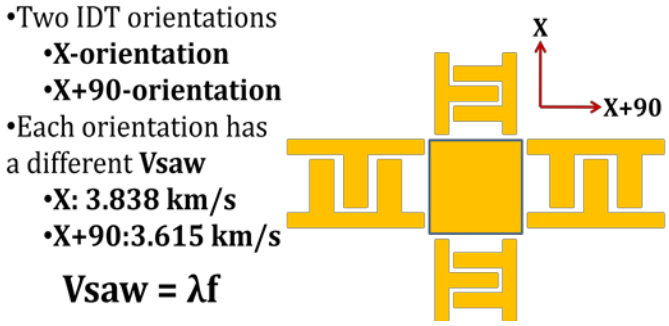

Figure 2 Orientation of IDTs on lithium niobate substrate.

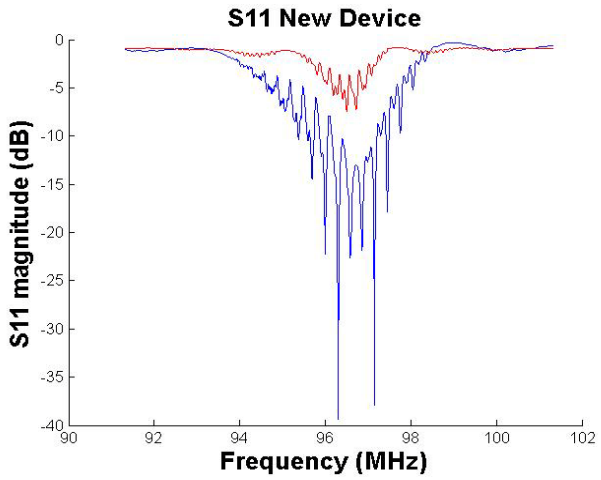

Figure 3. Graph of frequency responses for X-orientation (blue) and X+90-orientation (red) IDTs. Negative the magnitude indicates better the signal transmission.

Table 1. Frequency responses for devices fabricated at 50, 100, and 150 MHz

Frequency (MHz)	Wavelength (μm)	Wavelength (μm) X+90 orientation	Electrode Width (μm) X-orientation	Electrode Width (μm)	X Phase velocity (km/s)	X+90 Phase velocity (km/s)	X frequency (MHz)	X+90 frequency (MHz)
50	80	76	10	9.5			47.975	47.566
100	**40**	**38**	**5**	**4.75**			**95.95**	**95.132**
150	26	25	3.25	3.125	**3.838**	**3.615**	147.615	144.6

Once device fabrication and testing was completed, the IDT power supply system was designed and constructed. The system required that each component be capable of withstanding a minimum of 2 W, have an impedance of 50Ω, and permit the IDTs to powered individually.

Although system components were matched to 50Ω, the IDTs themselves were not. Since an IDT not matched to 50Ω would result in power loss, potentially compromising the system's ability to move droplets and resulting in damage to key electronic components, IDTs, a circuit board consisting of transmission lines, inductors, and capacitors was fabricated to match the IDTs to 50Ω while simultaneously transmitting the signal. Two solid state relay switches were also a part of the board to digitally control the activation of individual IDTs.

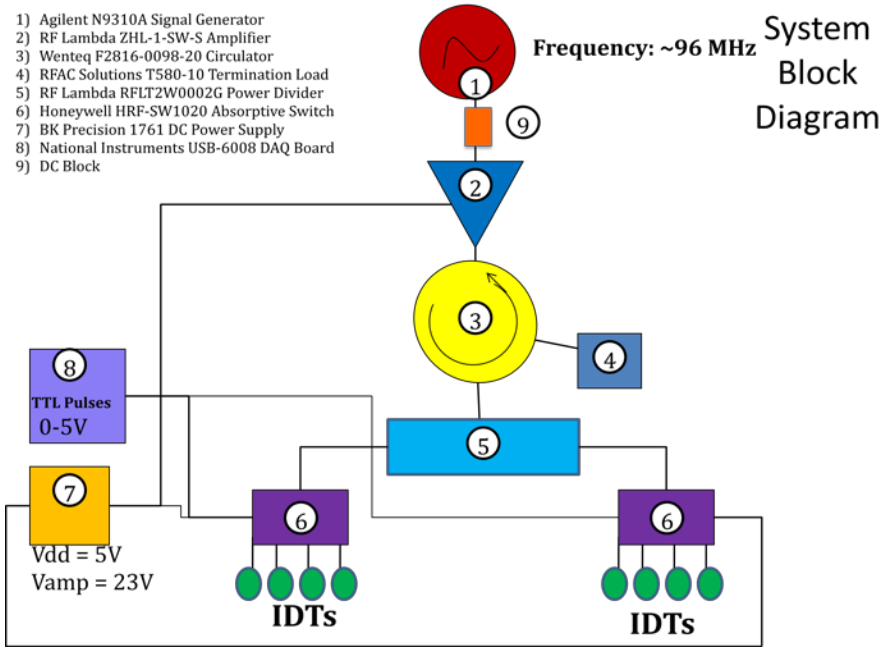

Figure 4 Block diagram for testing system.

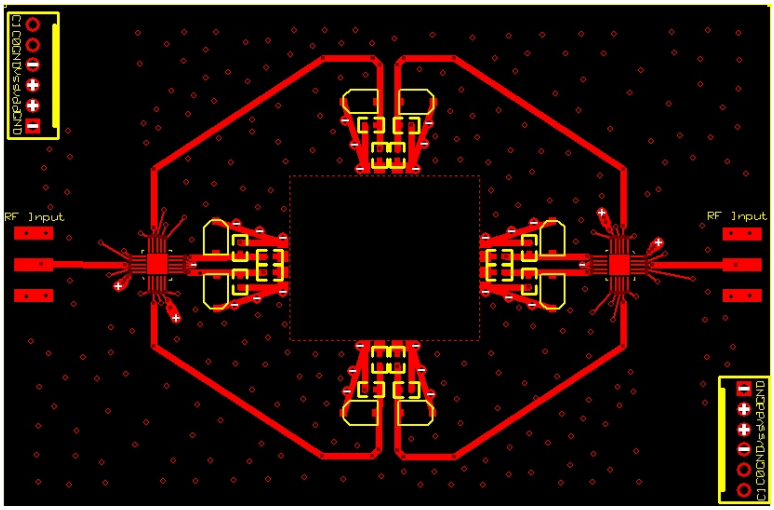

Figure 5 Layout of top layer of circuit boart used to match IDTs to 50Ω.

Once the system was tuned to an operating frequency of 96.824 MHz, it was demonstrated that droplets ranging from 0.1 µL to 0.5 µL could be moved along the gold transport surface. The transport surface was modified with alkanethiols to restrict the droplet's path of movement, forcing it to travel in a straight line.

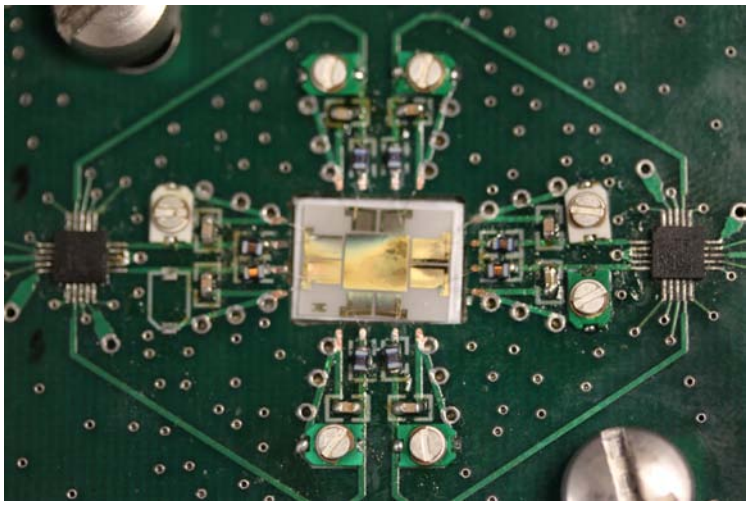

Figure 6 Final circuit board.

While SAW devices have been shown to move the microdroplets, heating the droplets can raise serious difficulties. Since the droplet volume is miniscule, evaporation occurs too quickly to determine if SAWs will have an effect on the germination of bacterial endospores. Two strategies will be combined to limit evaporation. First, a Peltier device built into the control board is now being used to reduce the temperature of the SH-SAW to below room temperature and limit evaporation due to heating. Second, a small chamber is mounted to the sensor chip to completely enclose the droplet(s) once it is directed toward the chamber.

Part IV. Opportunities for Training and Development

Tim Lyford is an M.S. student who has worked with Drs. Millard and da Cunha since September, 2009. Megan Harris is an M.S. graduate student in the Millard lab who began her work in September, 2009. Erin Gilbert is a Bioinformatics and Molecular Biology major (sophomore) at Rensselaer Polytechnic Institute who trained in the REU program in the Millard lab in summer 2011, working largely with Megan Harris. On June 17, 2010 Joshua Peimer completed a Masters of Professional Studies (M.P.S.) degree on this project as his Thesis Research. During the summer of 2011 Dr. Singer hosted Alois Cariou, a technical school exchange student from France. Alois was pursuing his D.U.T Biological Engineering Option A.B.B (Biological and Biochemical Analysis) at I.U.T of Quimper. During the summer of 2011 Dr. Singer mentored Michelle Dullinger, a B.S. in Biochemistry undergraduate who completed her capstone research project in his laboratory in the MBS department.

Introduction

A second no-cost extension for this project was requested during the latter part of Year 4 in order to allow completion of the project goals. Progress made during Year 5, the final year of the project, involved two linked areas of research. Each part is described in detail below. A third section describes student training in the final year of the project.

In summary, plasmids containing unique DNA sequences were introduced into *G. thermoglucosidasius* bacteria. These sequences were retained during sporulation and subsequent outgrowth. A number of different strategies for detection of multiple sequences on-chip by PCR were explored, the most successful being laminated Kapton-glass structures in which unique PCR primers and fluorescent probes were lyophilized. Rehydration and subsequent PCR have yielded some positive results, but more work will required to refine these techniques. The use of SAW devices for bacterial cell lysis prior to PCR analysis was developed and further applied to erythrocytes, expanding the range of applications of the technique. A number of undergraduate and graduate students received multi-disciplinary training over the course of the project.

Part I. Microsystem for Multiplexed DNA Analysis

Polymerase chain reaction: PCR reactions were prepared using 2x PCR Master Mix (Promega, Madison, WI), 0.5 µM of each oligo primer, 0.25 µM of duel-labeled oligo probe, <200 ng of template DNA, and 1mg/ml of bovine serum albumin. PCR was pre-formed in a Mastercycler Gradient Thermocycler (Eppendorf, Hauppauge, NY) for 2 min at 95°C, followed by 40 cycles of 30 s at 95°C and 1min at 50°C with a 4°C hold.

Modified qPCR fluorescent collection and analysis: PCR samples amplified using the dual-labeled oligo probe were analyzed using a different method than that of a standard qPCR system. ,After PCR was complete, samples of reactions that were performed in polypropylene tubes were collected in glass microcapillaries (Drummond, Broomall, PA) for imaging, while PCR reactions performed in glass-composite devices or sealed glass microcapillaries were imaged *in situ*. The lumen of the capillaries or device chambers were imaged using an upright epifluoresence microscope (Olympus BX51) and cooled CCD camera (SPOT RT3). Using imaging software (Metamorph, V7.1), average relative fluorescence intensity unit (RFU) measurements were made and fractional fluorescence change values were calculated as:

$$\text{Fractional fluorescence increase} = (RFU_{final} / RFU_{initial}) - 1$$

Thermocycler PCR*:* PCR reaction mixtures were prepared as described earlier. Reactions were analyzed using gel electrophoresis as well as fractional fluorescent increases.

Capillary PCR*:* PCR reactions mixtures were prepared as described in methods. Premixed PCR solutions were loaded into glass micro-capillaries (Drummond) using capillary action and sealed using optical adhesive #81 (Norland, Cranbury, NJ). Micro-capillaries were placed on thermocycler with an *in situ* adapter (Eppendorf) to promote even heating.

Device PCR: PCR reactions mixtures were prepared as described. Premixed PCR solutions were loaded into the device, initially by hand, and later through bonded-port connectors (LabSmith). Future devices will utilize these ports for attachment to microfluidic tubing (Upchruch) and a

syringe pump (LabSmith). Devices were placed on thermocycler with an *in situ* adapter (Eppendorf) to allow even heating. Devices were analyzed using fractional fluorescent increases. Capillary PCR reactions were run in parallel with device PCR as positive and negative controls.

Fabrication of devices**:** All devices were fabricated using a modification of a previously described protocol (Crews et al). Chambers were drawn with graphic design software (Adobe Illustrator) and sent to a cutting plotter (Graphtec Craft ROBO Pro), which cut the chamber patterns into 2-mil polyimide silicone adhesive double-sided tape (Caplinq-Kapton). Chamber sizes varied with the individual designs. Forceps and a stereomicroscope were used to weed-out the cut chambers from the tape. Prior to the assembly with tape, 1 mm diameter access holes were drilled through glass microscope slides with a diamond tipped flat-end drill using a mini drill press (Dremel). Slide glass was cleaned (Contrad 70), blown dry with nitrogen, heated to 120°C along with tape, exposed to a directed spark generator, and placed in a vise to facilitate bonding. The glass-composite device was sterilized by autoclaving. Bonded-port connectors (LabSmith) were added to devices using cyanoacrylate glue (LOCTITE Gel Control Super Glue) and allowed to cure for greater than 2 hr.

Glass Composite PCR Device**:** The detection of the unique DNA taggant was carried out using an in house fabricated polymerase chain reaction (PCR) chip array.The most successful device for the taggant amplification to data has been a glass composite design. Fabrication, as previously outlined by Crews et al., required cutting chamber shapes in a double-sided polyimide tape using a cutting plotter. The cut shapes were removed from the tape leaving behind the horizontal walls of the chambers. Sandwiching the tape between two glass microscope slides generated the top and bottom walls. Access to the chambers was done by drilling holes into the top slide glass prior to its application with the tape.

Uniform pressure facilitates bonding of glass composite devices**:** By applying uniform pressure on the glass composite using a vise and machined parallel bars, air bubbles between glass and adhesive were removed.

Fast fabrication: This technique of device fabrication allowed for rapid prototyping of chambers. Designs could go from conception to testing in less than an hour.

Tape when heated and spark-treated create a bond that resisted de-lamination: Since the liquid in the chambers was heated to near boiling it was important to ensure that the tape did not de-laminate from the glass. Using the fabrication method outlined by Crews et al. that heated the glass composited while a vice grip resulted in almost instantaneous de-lamination at elevated temperatures (Figure 1). Alternative a surface treatments with a site directed spark generator, often used for irreversibly bonding polydimethylsiloxane (PDMS) to glass, was also insufficient. However, the combination of these two methods by first heating the tape and glass followed by spark treating both surfaces resulted in a seemingly irreversible bond.

Glass composite devices can be autoclaved**:** The bond between tape and glass was strong enough to withstand steam sterilization. This step destroyed any contaminating DNA or nucleases acquired during fabrication.

Amplification of ADK in genomic DNA results in fractional fluorescent increase in polypropylene tubes and in microdevices**:** Previous experiments showed that the adenosine kinase gene (ADK) found in the genome of *G. thermoglucosidasius* showed a fractional

fluorescence increase in polypropylene tubes using a modified qPCR analysis method (Figure 2). This same amplification in glass composite devices showed 7-fold fractional fluorescence increase while a negative control amplification was essentially zero (Figure 3). In preliminary experiments, genomic DNA obtained through SAW lysis was amplified in a glass composite device and resulted in a 0.9-1 fractional increase in fluorescece, depending on the length of time the bacteria were exposed to SAW (Figure 4).

***Amplification of GtTAG from XL1-Blue(pGtTAG) DNA resulted in a 10 fractional fluorescent increase in polypropylene tubes and a 4 in devices*:** Using the pGtTAG plasmid DNA extracted from E. coli XL1-Blue the GtTAG unique DNA fragment showed a 10 fractional fluorescent increase in polypropylene tubes using a modified qPCR analysis method (Figure 5). This same amplification in glass composite devices showed a 4 fractional fluorescent increase while negative amplification showed a 0.5 increase (Figure 6).

***Labsmith Nanoports can be added to drilled access holes using cyanoacrylate**:* Adding bonded-port connectors seals the device for thermocycling and created a method whereby the device could be connected to other microfluidic systems (Figure 7). Use of a cyanoacrylate allowed for the re-use of port connectors cleaned with acetone.

Figure 1. De-lamination of fluid (blue) occurs at higher temperatures in stationary chambers unless heating and a spark-treatment occur during fabrication.

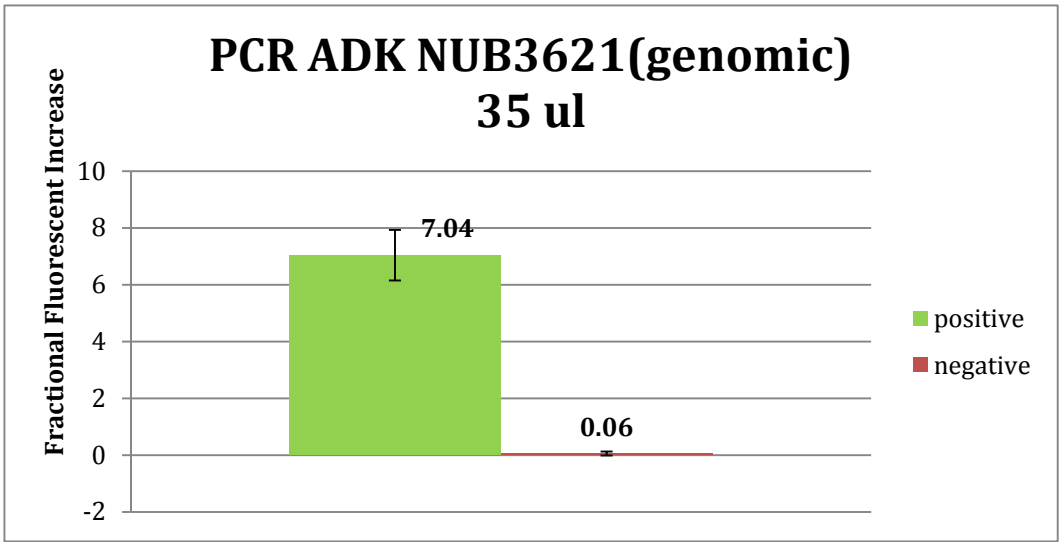

Figure 2. PCR of the ADK gene with NUB3621 isolated genomic DNA in polypropylene tubes. Positive corresponds to amplification of the ADK gene, while negative corresponds to the amplification of a gene not found in the NUB3621 genome.

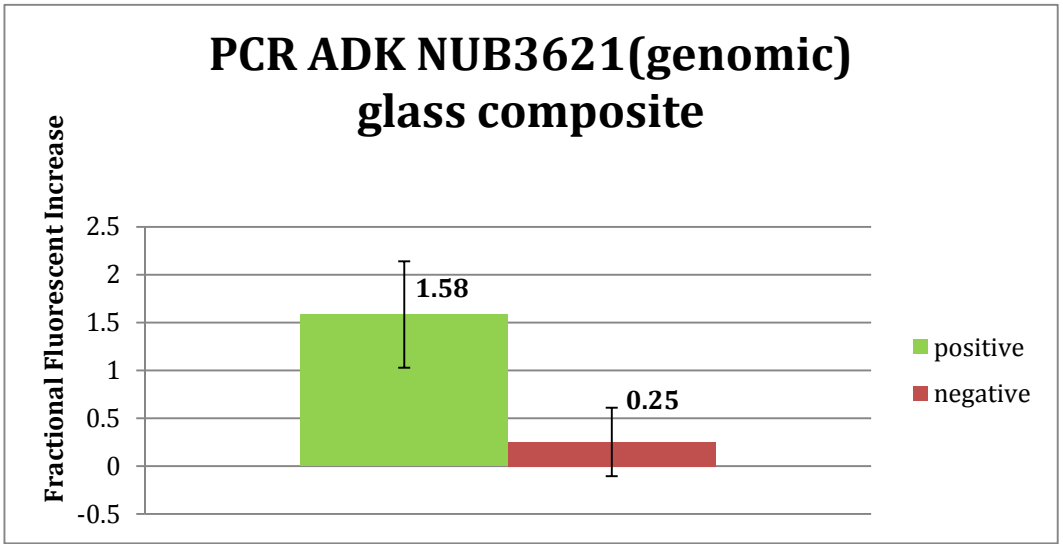

Figure 3. PCR of the ADK gene with NUB3621 isolated genomic DNA in a glass composite device.

Positive corresponds to amplification of the ADK gene, while negative corresponds to the amplification of a gene not found in the NUB3621 genome.

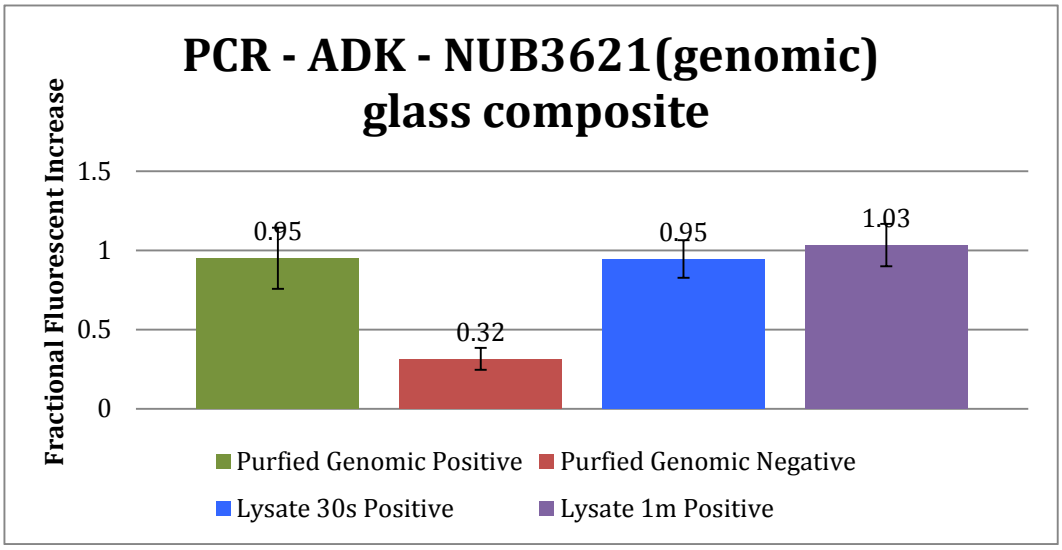

Figure 4. PCR of the ADK gene with NUB3621 isolated genomic DNA in polypropylene tubes. Positive corresponds to amplification of the ADK gene, while negative corresponds to the amplification of a gene not found in the NUB3621 genome. Lysate corresponds to samples using template DNA was obtained through SAW lysis methods.

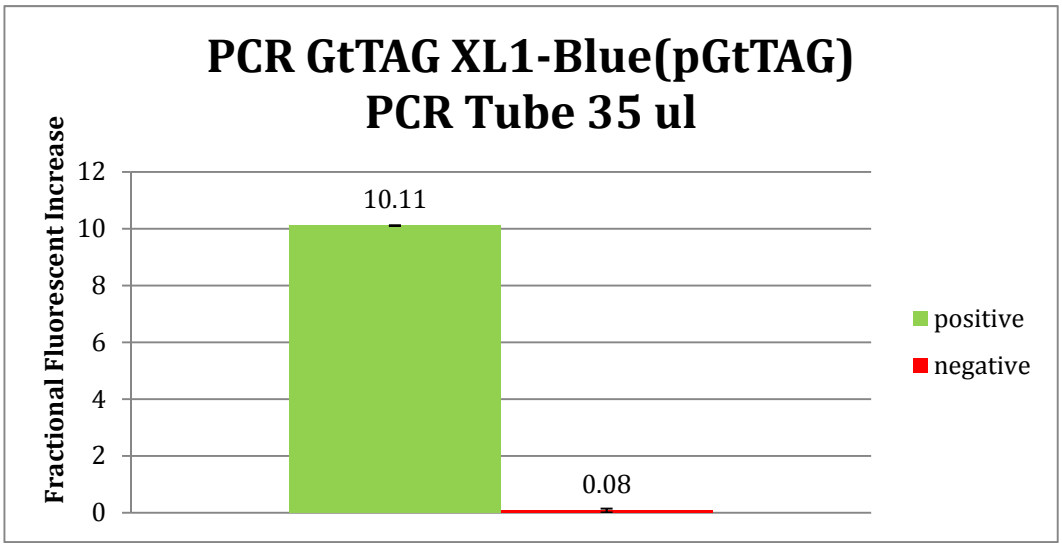

Figure 5. PCR of the GtTAG fragment with XL1-Blue(pGtTAG) in a thermocycler. Positive corresponds to amplification of the GtTAG fragment, while negative corresponds to the amplification of a fragment not found in the pGtTAG.

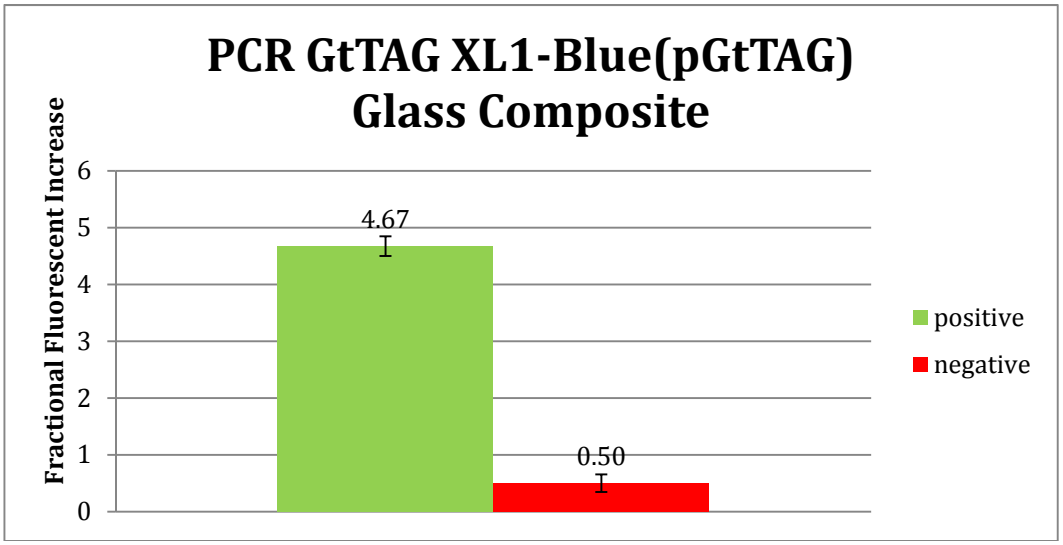

Figure 6. PCR of the GtTAG fragment with XL1-Blue(pGtTAG) in a glass composite device. Positive corresponds to amplification of the GtTAG fragment, while negative corresponds to the amplification of a fragment not found in the pGtTAG.

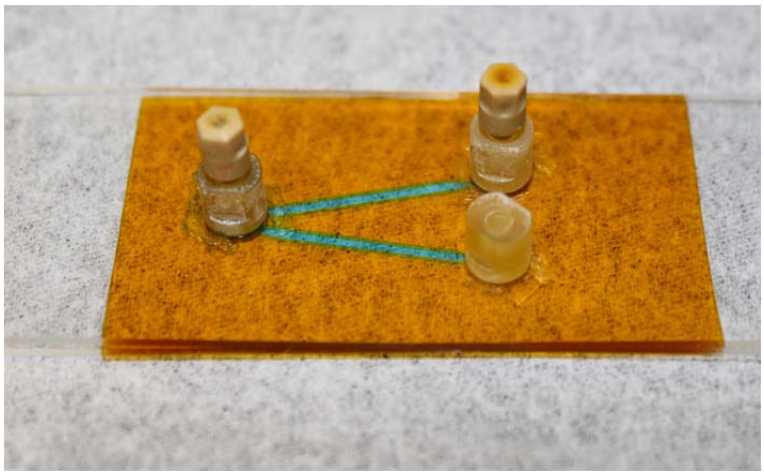

Figure 7. Sample glass composite device with bonded ports.

Part II. SH-SAW Platform for Bacterial Cell Lysis

Growth and Cultivation of Bacteria

The growth rate of *Geobacillus thermoglucosidasius* was investigated by undergraduate researcher trainees to determine when the bacteria entered the logarithmic growth phase.

Samples were measured hourly using a UV/Vis spectrophotometer at 600 nm to obtain an absorbance value. A growth curve was generated using the measured absorbance values (Fig.8). Cells were also counted to determine cell concentration, allowing for correlation between absorbance and cell concentration (Fig.9).

Students were also tasked in plating bacteria on sporulation agar medium and were also responsible for preparing agar plates and plating bacteria for the purpose of cell counts. Each undergraduate student gained substantial experience in basic microbiological techniques.

Bacterial Cell Lysis

An important outcome of the project was the development of a technique to lyse the bacterial cells. The technique allows for lysis without the use of chemical additives and can be integrated into portable DNA detection systems. A surface acoustic wave (SAW)-based device was designed and fabricated for droplet movement and lysis,. SAW technology is promising for further development of miniaturized lysis systems due to low power requirements needed for device operation

SAW Device and Experimental Setup: The SAW device used in this work was fabricated on Y-128° LNO wafers by depositing a 10 nm Cr adhesion layer, followed by a 150 nm Au layer. The device was designed to have delay lines operating along both X and X+90° orientations, as seen in Fig. 4. In this work the X+90° orientation was selected. Split-finger interdigitated transducers (IDT) with 260 electrodes of width 4.71 μm; mark-to-space ratio 1:1; and acoustic aperture of 754 μm were fabricated. The IDTs were matched to 50Ω using a series-L and shunt-C matching circuit, after which the measured $|S_{21}|$ was -5.9 dB and $|S_{11}| \approx |S_{22}|$ were -22.5 dB at an operating frequency of 96.6 MHz.

Prior to testing, the gold surface located between the two IDTs was chemically treated to be hydrophobic by immersing the wafer overnight in a 5 mM solution of heptadecafluor-1-decanthiol (Sigma-Aldrich, USA) in 100% ethanol (EtOH) and washed with 100% EtOH prior to experimentation. An increase in hydrophobicity aided in droplet formation on the testing surface, eliminating the spreading of water over the wafer's surface and onto the IDTs.

Surface loading of the SAW device with a 10μL droplet (containing bacteria) resulted in an increase in transmission loss, with $|S_{21}|$ varying from -5.9 dB to ~ -40 dB, indicating that a major fraction of the acoustic energy was transferred into the liquid. This device was designed with the intention of transmitting most of the RF energy into the bacterial suspension, and therefore neither acoustic absorbers nor a tilted crystal end was employed. As a result, ripples due to reflections on the crystal borders could be measured in the transmission response, but once the droplet was in place most of the energy was transferred into the cell suspension, resulting in severe attenuation of the ripples.

An Agilent N9310A signal generator was used to apply the RF signal to the system. The signal was amplified to 2W using an RF Amplifier (Lambda ZHL-1-SW-S,USA). After passing through a circulator (Wenteq F2816-0098-20, USA), the signal was split using a power divider (RF Lambda RFLT2W0002G, USA) and transmitted through LC-matching circuits to the two opposing IDTs, both of which were used as input ports (Fig. 9). For bacterial disruption tests, 1W (measured with an Agilent N1912A power meter) was applied to each IDT port.

The temperature at the surface of the SAW device was controlled by mounting the cut wafer on a detachable aluminum block that could be disconnected from the circuit board after each test to allow for device cleaning (Fig. 11). The aluminum block was held in contact with a Peltier device capable of cooling or heating the block to a controlled temperature, thus allowing the temperature of the SAW device to be regulated from 5°C to 95°±1°C. A thermistor was affixed to the aluminum block to provide feedback to a temperature controller (Oven Industries #5R7-001, USA).

Prior to applying power to the IDTs, the surface temperature was adjusted and a 10 µl droplet of bacterial suspension pipetted onto the gold surface between the IDTs. A removable plastic cover was placed over the droplet to reduce evaporation loss and the RF signal was applied to the droplet for up to 8 min. After RF exposure the sample was collected using a pipette and diluted in sterile nanopure water to 100 µl. Any fluid loss due to evaporation was corrected in the final dilution. After each test the SAW device was cleaned thoroughly by sequential washing in Contrad detergent, water, and 70% EtOH to remove any DNA carryover. In order to discriminate SAW-mediated effects from heating alone, the extent of cell disruption by SAW exposure was contrasted with experiments in which the substrate surface was simply heated or cooled in the absence of the SAW RF signal.

***SAW Device Characterization*:** S-Parameter measurements were recorded to characterize the device before implementation in the lysis system. |S21| was recorded and gated. Three ripples in the gated response (Fig. 12a), spanning from approximately 95.46 MHz to 96.76 MHz, are due to waves reflecting from the edge of the cut wafer. The addition of a droplet eliminates the response and decreases the |S21| to ~-40 dB, indicating that most of the acoustic energy is absorbed by the droplet. Measurements of |S11| and |S22| before and after the addition of the droplet were recorded and compared. Pre-droplet measurements further confirm that multiple reflections occur due to the waves reflecting off the border of the cut wafer; adding a droplet to the surface results in the deadening of the reflections, resulting in a smoothing of the |S11| and |S22| responses (Fig. 12b). The device's response in the time domain from approximately 1.3 µs to 4.1 µs is likely the result of a wave traveling from the midpoint of the IDT to the wafer border and back (Fig. 12c). The returning wave also generated a bulk acoustic wave response from 2.7 µs to 3.6 µs; reflections from the border are seen from 6.4 µs to 8.2 µs. When present, a droplet eliminates these responses due to acoustic wave absorption.

***Lysis Conditions for Comparison*:** Two treatments were compared with SAW exposure: ultrasonic probe and heat. These tests were conducted by an undergraduate summer REU student and were vital for (1) determining the lysis efficiency of the SAW device and (2) discriminating lysis due to SAW from that resulting from heating.

To determine the efficacy of SAW treatment with respect to lysis of NUB3621, 100% lysis was accomplished using an ultrasonic probe (Fisher Scientific Sonic Dismembrator Model 100 with 3 mm probe tip, 100W, USA). Bacteria were disrupted in 1 ml of water at 5°C. Samples exposed

continuously to sonication were collected over 480 sec. The concentration of released DNA remained constant after 240 sec exposure (Fig. 10). This DNA yield was defined as the level corresponding to 100% lysis. Microscopic observation using differential interference contrast (DIC) microscopy confirmed the lysis of all bacteria (Fig. 10, inset).

Before proceeding with the lysis experiments using the SAW device, tests were performed to determine the effect of temperature alone on cellular integrity (Fig. 13). For surface temperatures ranging from 5° to 55°C and 95°C, less than 2.5% of the bacteria were disrupted after 480 sec.

Lysis with the SAW device: Bacterial lysis experiments using the SAW device were performed at 5°, 35°, and 55°C. Fig. 13 shows that cell lysis by the SAW device is more pronounced at higher temperatures. At 35°C more than 30% of the cells are lysed, compared with only 1% at 35°C without SAW input. At 55°C cell lysis using SAW was accelerated, however droplet evaporation for the experimental setup limited the useful test time to 60 sec. Overall, the data indicate that when compared to elevated temperature alone, the SAW exposure markedly increases the rate and extent of DNA release. The rate of release of DNA is consistent with rapid sample analysis. In addition, bacterial lysates generated by acoustic wave exposure at 5°C and 35°C can be subjected to protein analysis, since they have not been subjected to elevated temperatures that might denature proteins.The percentage of cells lysed by the SAW device is sufficient for DNA detection and analysis.

Post-SAW Lysis Detection of DNA Sequences by PCR: DNA samples collected from SAW-lysed bacteria were analyzed by qPCR to determine whether specific genomic DNA sequences were not altered by the SAW processing and thus could be detected post-lysis. Higher concentrations of target DNA released by the SAW-lysis procedure require fewer amplification cycles, reducing sample analysis time. Shown in Fig. 14 is a representative plot of the collected data for samples exposed to SAW for 60 sec and held at temperatures of 5°, 35°, or 55°C. An untreated sample and 100% lysed sample were also included for comparison. The untreated sample required 23 cycles before detection, while SAW-treated samples required 22 cycles (5°C), 18 cycles (35°C), and 17 cycles (55°C). The sonic probe, which requires at least 240 sec for 100% lysis, required 16 cycles for detection of released genomic DNA.

Sixty seconds of SAW exposure at 55°C reduced the number of PCR cycles required by 6 cycles, one cycle more was required by the 100% lysis sample obtained with sonication. qPCR verified that specific genomic DNA sequences released by SAW treatment are amplified and could potentially be used in DNA-based sensing applications. The SAW device is therefore an effective method for disrupting vegetative NUB3621 bacteria and should be applicable to many other organisms and cells. This technology is promising for portable medical diagnostic devices requiring cell lysis for sample analysis.

Red Blood Cell Lysis

In addition to bacterial cells, the SAW device was tested as for its ability to lyse erythrocytes in whole bovine blood. An undergraduate trainee was funded through a cooperative arrangement with Instrumentation Laboratories, Bedford, MA, to conduct the tests, exposing RBC for up to 240 sec. A Peltier device was used to maintain the surface temperature at 35°C for the tests. Hemoglobin release was measured after each test an absorbance value at 540nm. The absorbance value was compared to a 100% lysis standard using a chemical treatment of TX-100

to determine lysis efficiency. DIC microscopy using a 40x objective lens was used to verify cell lysis.

In addition to SAW exposure at 35°C, cells were also heated. The initial temperature was 25°C. After 240 sec, the temperature was increased by 5°C up to a maximum temperature of 60°C.

Results of heating: From 25° to 50°C, no visible change was observed in the RBC. Increasing the temperature to 55°C resulted in the RBC rapidly disappearing, and. membrane ghosts becoming visible, indicating cell lysis.

RBC Lysis using Surface Acoustic Wave Device: Using a set temperature of 35°C, the SAW device was able to lyse 100% of bovine erythrocytes in whole blood within 240 sec of exposure. Approximately 37% of cells were lysed within 120 sec of exposure, and after 180 sec of exposure, ~93% of all cells had lysed (Fig.15). Cells were examined using DIC microscopy to confirm lysis (Fig.16).

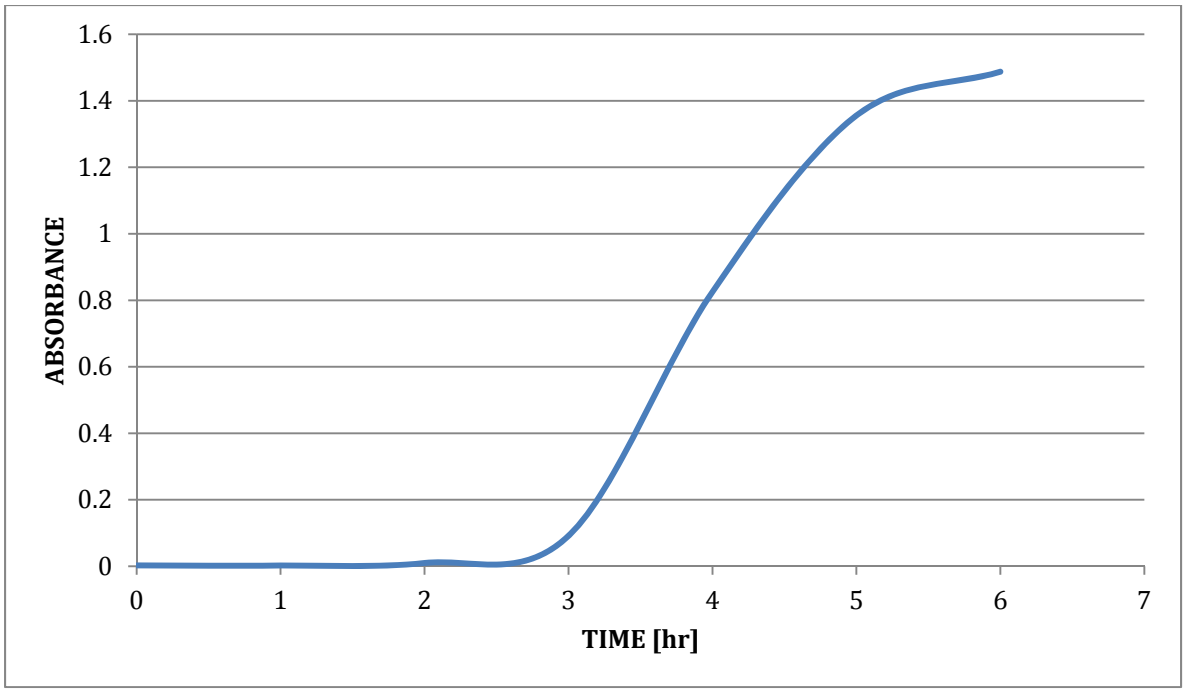

Figure 8. Growth rate of *G. thermoglucosidasius*.

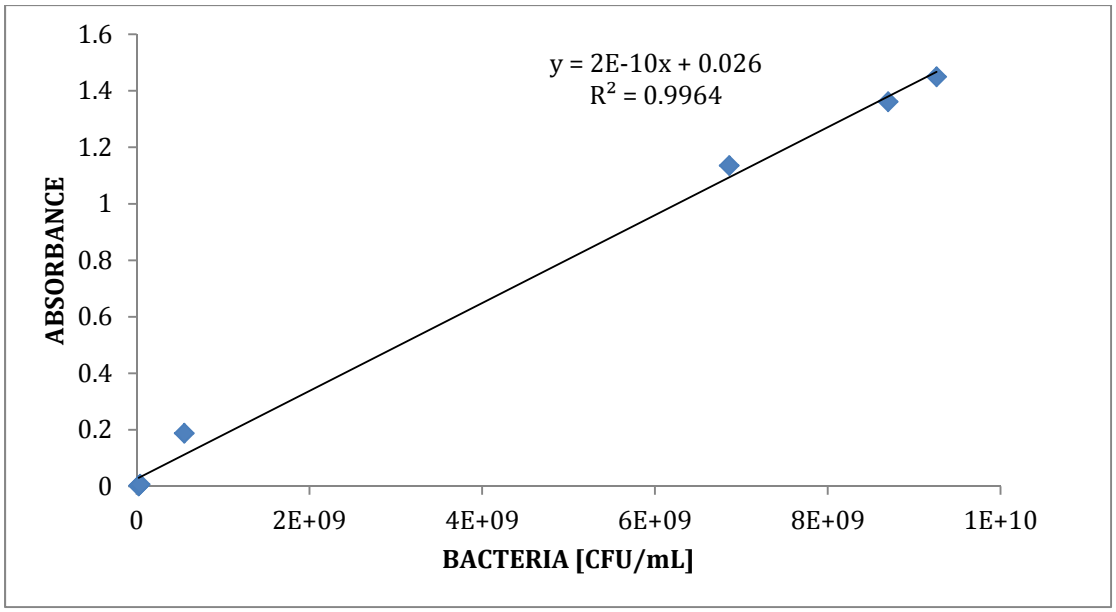

Figure 9. Optical absorbance as a function of *G. thermogluocosidasius* cell density.

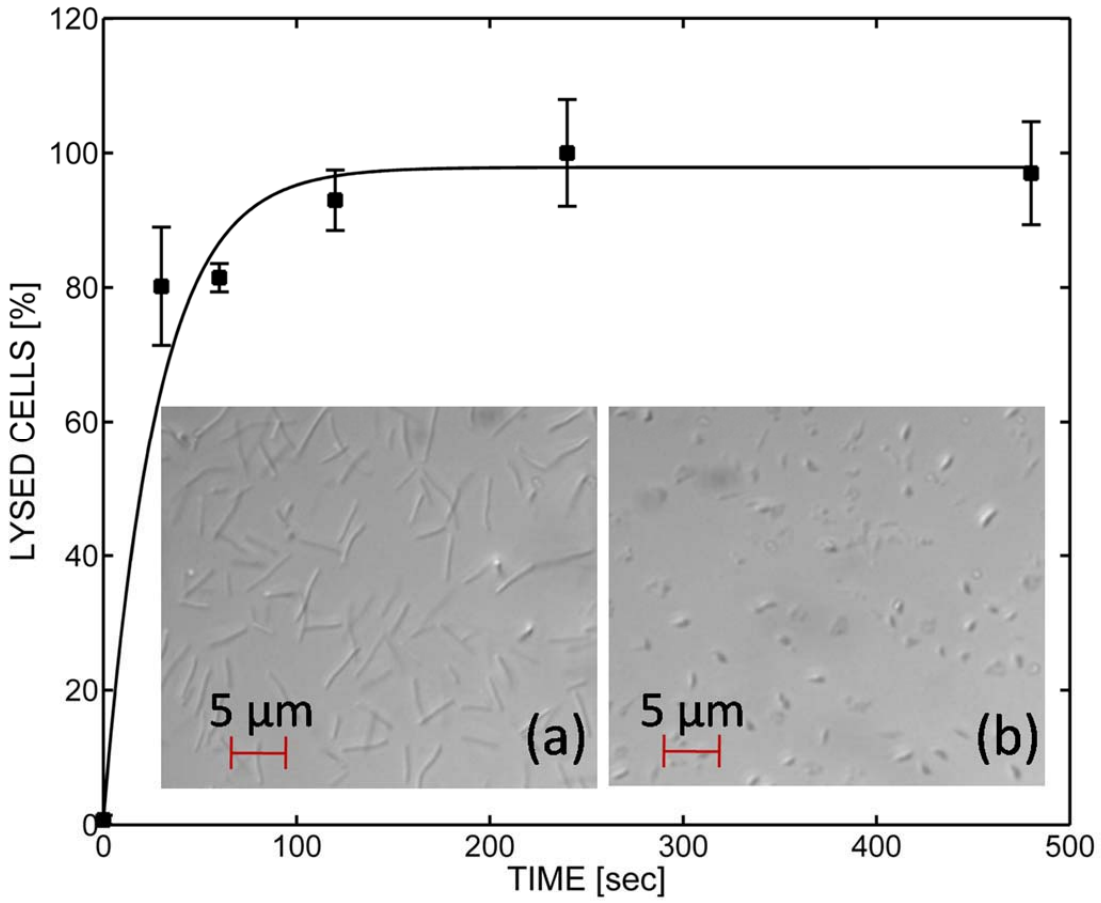

Figure 10. Percentage of cells lysed using the ultrasonic probe. Images were taken before (a) and after (b) probe treatment to verify the extent of cell lysis indicated by free DNA fluorescence measurements.

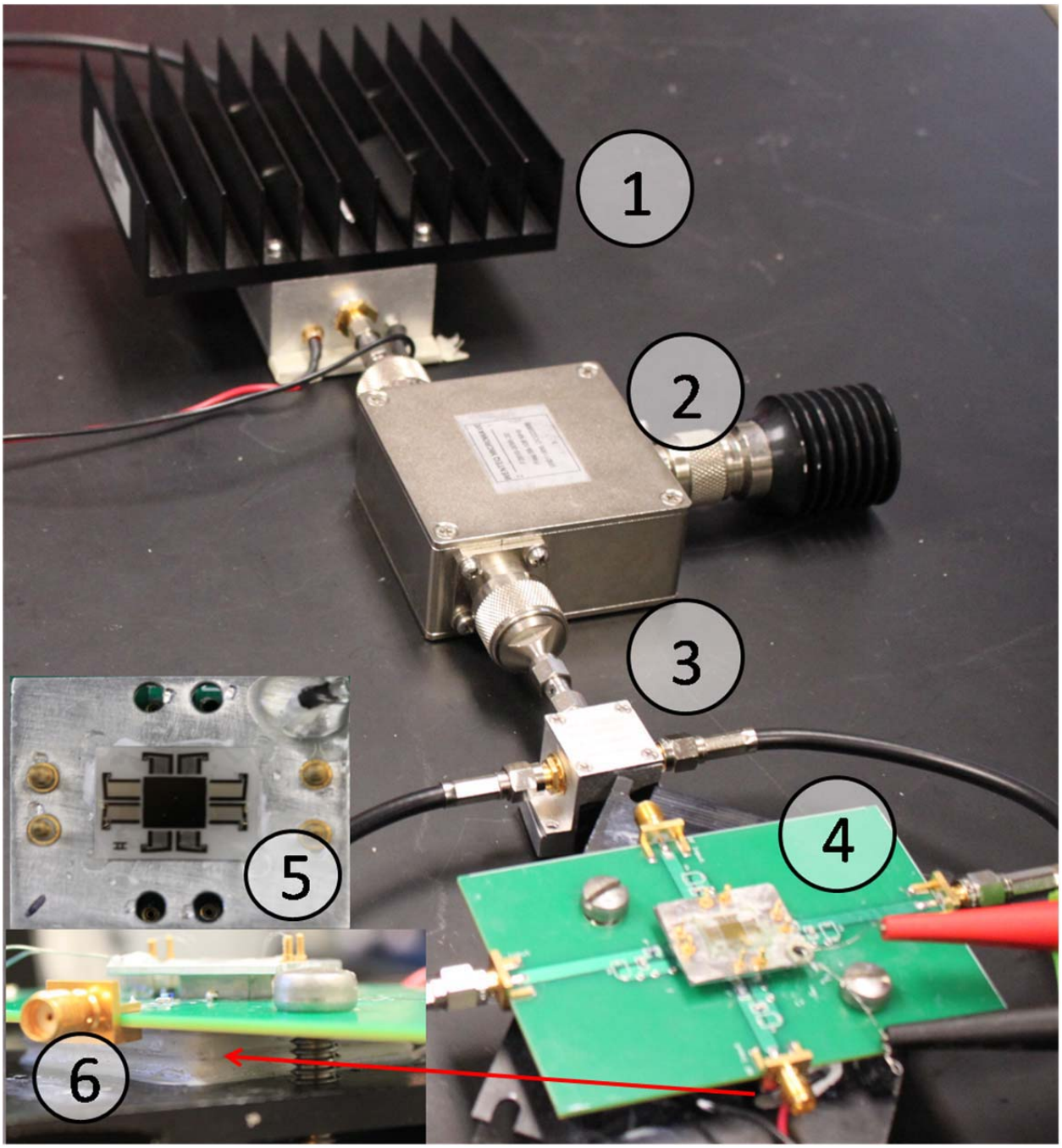

Figure 11. An RF signal is applied to the system and amplified to 2W using a DC-powered RF amplifier (1). The signal passes through a circulator (2) and a power splitter (3). Each signal line is matched using a series LC-shunting network (4) and RF signal is transmitted to the SAW device (5). The SAW device is attached to an aluminum block in contact with a Peltier device (6).

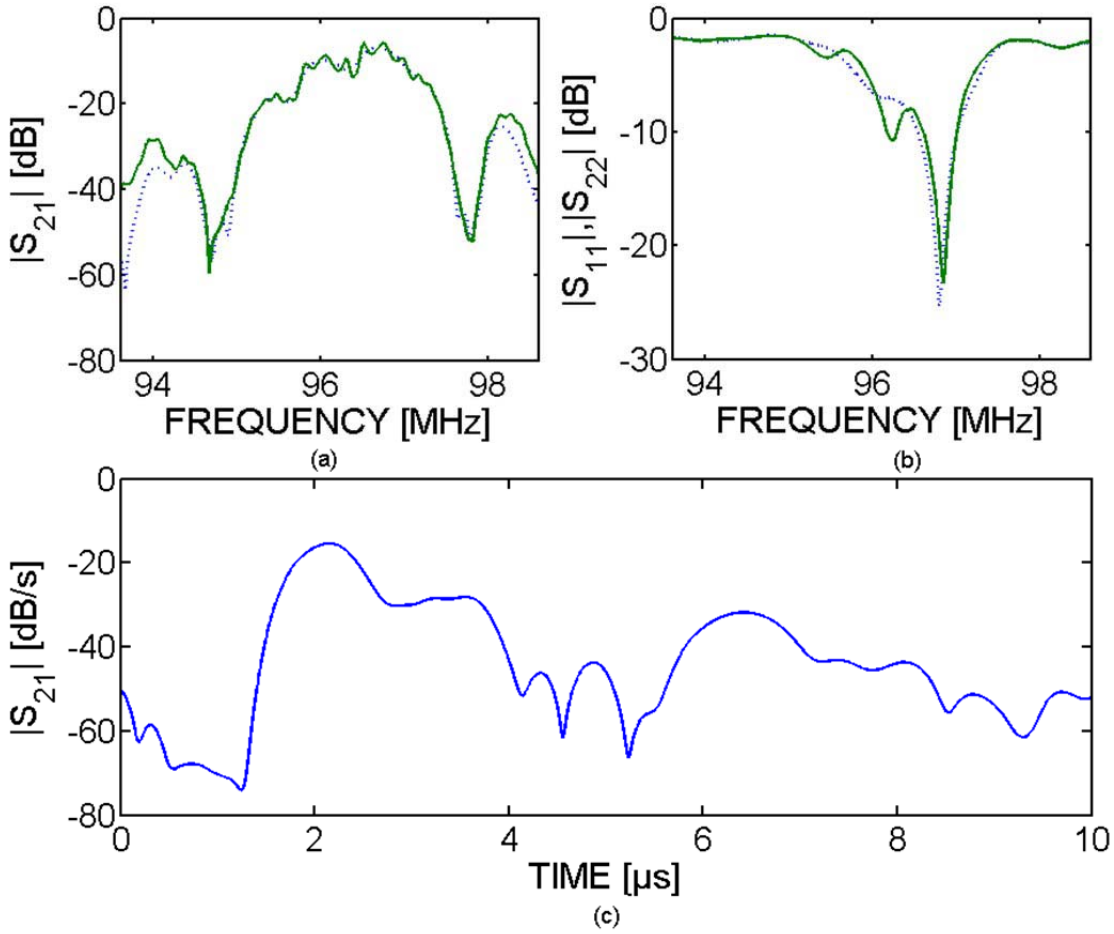

Figure 12. S-Parameter characterization of SAW device's X+90°orientation fabricated on Y-128° lithium niobate. |S21| is shown before (solid) and after time gating (dash) (a), showing ripples that are due to the signal reflecting of the LNO wafer's border. |S11| (solid) and |S22| (dash) were measured after the addition of a droplet to the surface (b). The signal was gated from 0 to 5.5 μs (c).

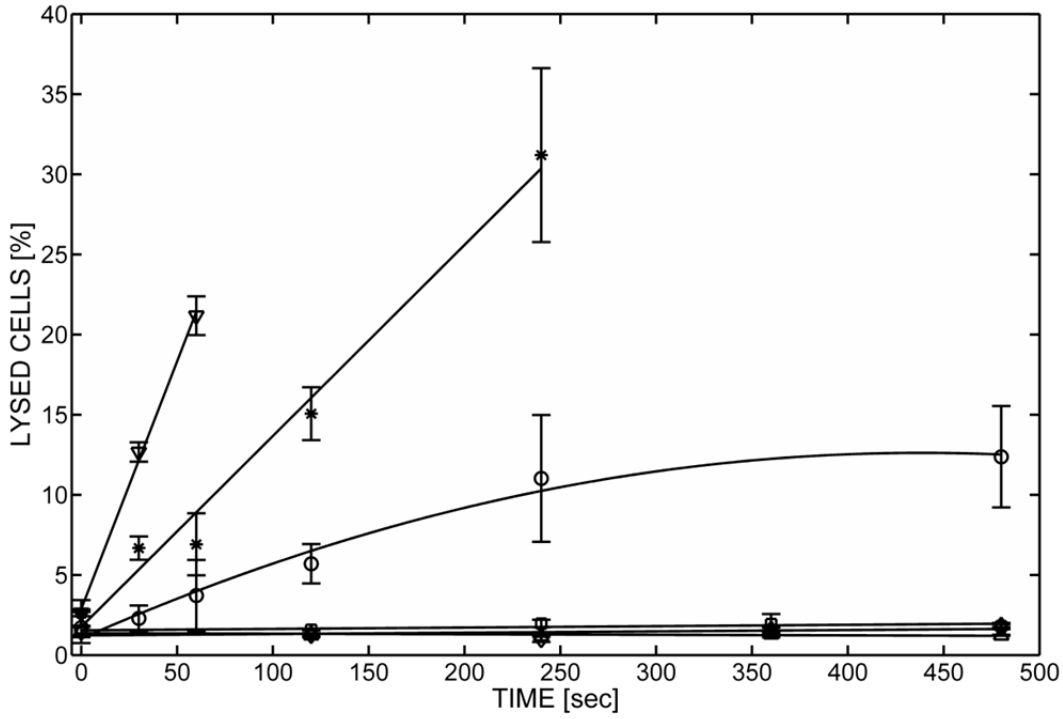

Figure 13. Lysis efficiency of surface acoustic wave device (1W output per IDT) coupled with heat. SAW + 5°C (o), SAW + 35°C (*), and SAW + 55°C (▽) are shown above. Tests to determine cellular integrity at 5°C (▽), 35°C (◊), and 55°C (□) are also shown for comparison. Lines are used only to show the trend of the points and have no statistical significance.

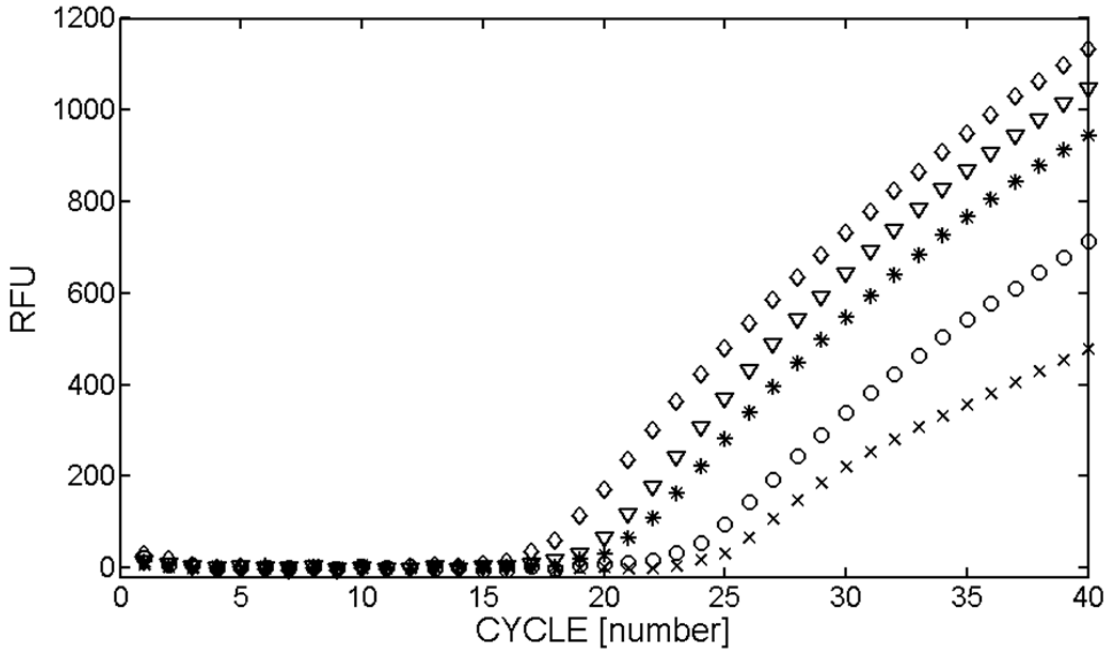

Figure 14. qPCR results of experimental samples obtained from SAW-exposed cells. Samples held at 5°C (o), 35°C (*), and 55°C (□) and exposed to SAWs for 60 sec are shown above with a 100% lysed sample (◊) for comparison. DNA amplification is indicated as relative fluorescence units (RFU). A sample of organisms not exposed to SAW was also analyzed (x).

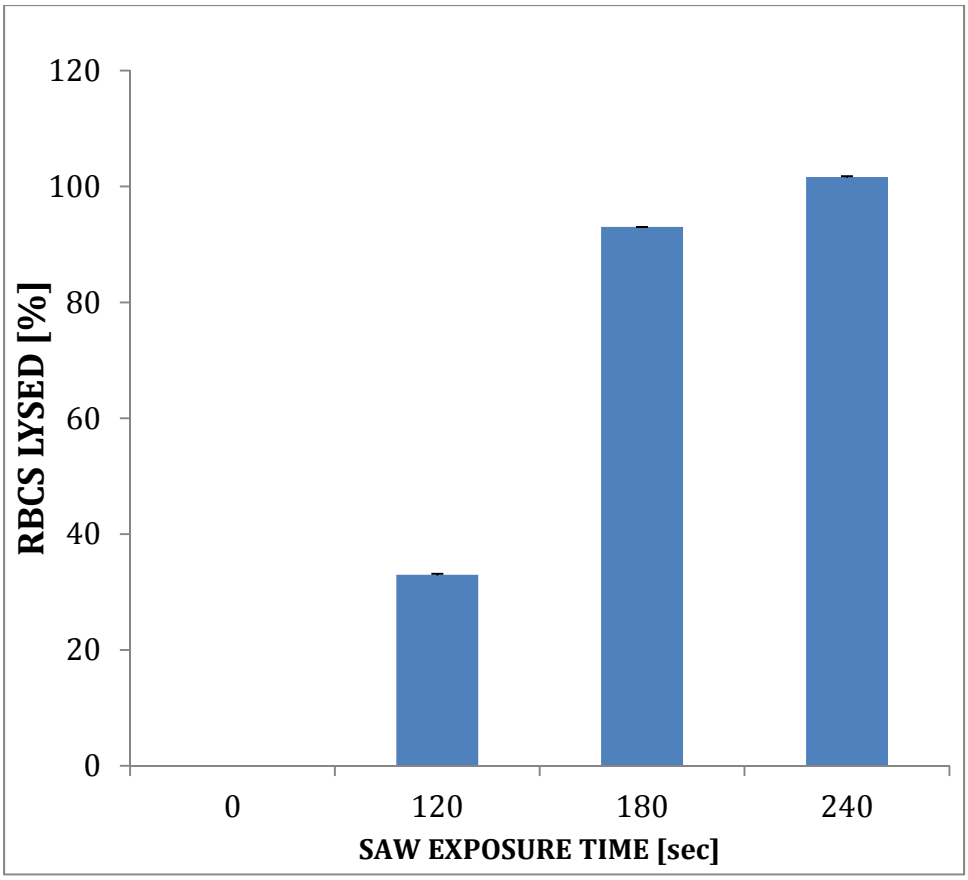

Figure 15. Effect of SAW exposure time on RBC lysis.

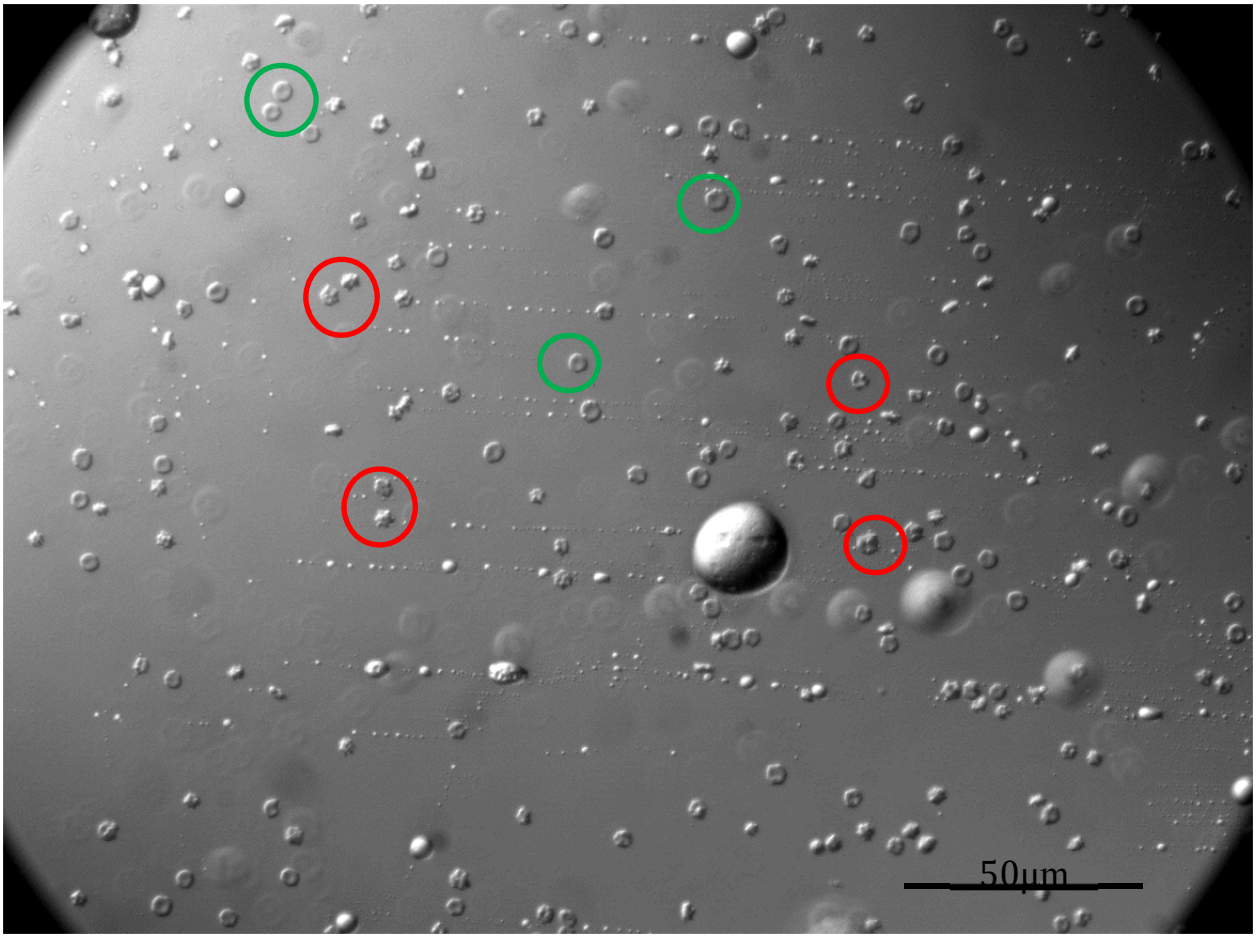

Figure 16. SAW-treated sample after 120 sec exposure. Normal RBC (green), and fully and partially disrupted RBC (red) are shown for comparison.

Part III. Opportunities for Training and Development

Tim Lyford is an M.S. student who has worked with Drs. Millard and da Cunha since September, 2009. Megan Harris is an M.S. graduate student in the Millard lab who began her work in September, 2009. Kepra McBrayer is an Electrical Engineering major (senior) at the University of Evansville, IL who trained in the REU program in the Millard lab in summer 2012, working largely with Timothy Lyford. During the summer of 2012 Dr. Singer mentored Alexis Quintillani, an undergraduate in the MBS department.

ACTIVITIES

MAJOR RESEARCH AND EDUCATION ACTIVITIES OF THE PROJECT

In the third year of funding we have focused our research efforts on three aspects of the current project: (1) alternative methods for genetic engineering of *Geobacillus thermoglucosidasius* NUB3621 required to generate transformed bacteria with unique DNA taggant sequences and genetic elements to permit selection, (2) molecular techniques and devices for multiplex on-chip PCR detection of DNA taggant sequences, and (3) the creation of SAW-based systems devices and instrumentation for droplet movement and mixing, and acceleration of endospore germination and vegetative cell lysis. The educational goals have been: (1) to identify highly motivated undergraduates and graduate students who are interested in pursuing multidisciplinary training in microbiology/molecular biology and engineering, and (2) to involve current and new students in various aspects of the project and promote regular dialog between students from different fields. We have been successful in introducing undergraduates to research through this project and have recently attracted several high quality graduate students to work in this area with us.