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A MICROFLUIDIC ACOUSTIC TRAPPING PROTOTYPE FOR RAPID PROCESSING OF SEXUAL ASSAULT EVIDENCE

DOJ AWARD NO. 2013-NE-BX-K027

Kerui Xu, Charlie Clark, Kimberly Jackson, Anchi Tsuei, Orion Scott and James Landers

Abstract

Forensic laboratories are overwhelmed with sexual assault DNA evidence that requires processing, thus exacerbating the need for a streamlined process that delivers high quality results with reduced cost and analyst time. The currently utilized differential extraction (DE) methodology, while effective, is labor-intensive, time-consuming, presents a higher risk for contamination, and infuses significant inefficiency to the overall workflow of forensic evidence processed in many laboratories. While moderate-to-high throughput systems have been developed (e.g., robotic 96-well plate) with conventional DE chemistry, the cost can be prohibitive for many laboratories. Consequently, a need existed for methods with reduced analysis and analyst time, to deconvolute 'mixed STR profiles' (perpetrator + victim DNA). Microfluidics was an attractive option for this as is combines low volume chemistry with minimal reagent and sample consumption, while in an automated closed (no intermediate sample transfer) system that can potentially be interfaced with a fully-integrated microfluidic STR profiling system [see Le Roux et al., Integrated sample-in-answer-out microfluidic chip for Rapid Human Identification by STR analysis. 2014. Lab Chip 14:4415–4425].

NIJ funding in 2006 allowed us to carry out a Phase-1 proof-of-principle effort on a high risk approach termed *acoustic differential extraction (ADE)* - a method that exploited acoustic energy to trap and isolate intact sperm cells in a sea of female cell lysate. Additional (non-DOJ) funding in 2009 allowed a Phase-2 effort with research focused on instrument/microfluidic device engineering and piezo-transducer (PZT) physics, to the point where we felt we were able to address forensically-relevant samples. As a result of this, an NIJ-funded Phase-3 effort allowed us to develop the *SONIC* system (***S**ex **O**ffender **N**odal **I**solation of **C**ells*) **–** a microfluidic system that couples the basic chemistry in current differential extraction (DE), with the direct isolation of sperm cells via acoustic trapping. The SONIC system developed in this effort is a generation-1 (*SONIC Mach-1*) system that is not productized. However, is has been engineered and built by a PhD-level Mechanical Engineer (with 7 years of industrial experience), thus, engineered well enough to be placed in forensic labs for testing and, if desired, easily productized. The system is composed of a microfluidic chip that is used for isolation of the male fraction from a sample, along with the hardware that facilitates the microfluidics under the control of a customized software. The prototype uses a laptop graphical user interface, where the user can input the necessary information needed for executing acoustic trapping. With this system, we have successfully demonstrated the isolation of adequate male fraction from mock samples where the female:male DNA ratio is as high as 100:1.

1. EXECUTIVE SUMMARY

The backlog of samples awaiting forensic DNA analysis, both casework and convicted offender or arrestee, continues to be a significant social problem in the US [5,1]. Despite the technological capabilities of forensic laboratories, a recent summary from 153 labs reporting end-of-year casework backlogs, indicate that >150,000 samples await DNA analysis, with greater than half the labs reporting turnaround times of four months (or less) with some reaching as far as 9 months [6,2]. A sizable subset of the samples comprising the backlog involve sexual assault evidence (>27,500 of the ~150,000 samples), where roughly 40% of these samples contain DNA evidence and require timely analysis for effective prosecution of suspected perpetrators [6,7 2,3]. There has been a realization that backlog reduction will not likely come from increasing the number of analysts, but from technology improvements that provide automation, decreased labor requirements and enhanced throughput.

With sexual assault evidence, obtaining a full, single-source DNA profile is dependent on the efficient separation of sperm cells from female epithelial cells or lysate. Differential extraction (DE) is the most widely-used method of choice, employing proteinase K and an anionic detergent to elute material from a cotton swab collected from the victim, selectively lysing epithelial cells, leaving sperm cells from the perpetrator intact. It also involves numerous sample handling and centrifugation steps, leading to the potential for contamination and loss of valuable sample. In addition, it can suffer from a lack of separation specificity, with carryover from the female DNA sometimes seen in the male fraction.

An initial round of NIJ funding (2006-DN-BX-K021) allowed for a Phase-1 proof-of-principle of a 'high risk' concept that could provide an alternative approach for DE - we termed this *acoustic differential extraction (ADE)*. This exploited acoustic energy to trap intact sperm cells in a sea of female cell lysate, ultimately isolating the male and female fractions. That funding evolved ADE from concept to a stage where we could demonstrate that sperm cell trapping could be accomplished - this was described in a publication in 2009 [9]. Additional (non-DOJ) funding allowed us to progress the technology (as a Phase-2 effort) with research focused on instrument/microfluidic device engineering and PZT physics, to the point where we felt we were able to address forensically-relevant samples.

Platforms that exploit fluid flow and chemical/biochemical reactions in the microfluidic regime offer a number of characteristics that address drawbacks of the standard DE method. Immediate benefits include reducing: 1) sample and reagent volumes, 2) contamination in a 'closed system', 3) the number of sample transfer steps and, 4) potential sample loss [5-11]. While several cell sorting techniques have been demonstrated on microfluidic devices, the use of acoustic trapping is most applicable to DE. With *Acoustic Differential Extraction* (ADE), low power acoustic energy is applied by a PZT to a fluid-filled microchannel generating a standing acoustic wave that creates low pressure zones capable of trapping particles in a size-selective manner [12]. We demonstrated preliminary success with ADE showing rapid extraction of sperm cells out of lysate from a swab containing epithelial and sperm cells.

The current project (2013-NE-BX-K027) built on efforts between 2009-2012 (funded but not by NIJ). In that time-frame we addressed some of the more detailed aspects of the trapping process including the acoustics-microchip interface, acoustic energy delivery to the trap zone, sample delivery methods and male/female fraction collection. Having worked through these fundamental challenges, the stage was set to address residual trapping parameters that needed to be optimized before testing on *bona fide* forensic samples. These parameters included: 1) *Sample delivery flow rate for sample loading in <10 minutes;* 2) *improved cell trapping capacity by setting multi-node trapping zones*; 3) *improved sperm cell trapping efficiency with low sperm cell number samples (total cells <100) ;* 4) *the effect of non-sperm cell constituents in the sample on trapping*; 5) *pre-chip sample preparation for optimal trapping*; 6) *post-SONIC sample preparation for direct-to-PCR analysis*; 7) *trapping sperm cells from mock samples with varied female:male cells ratios;* and 8) *placement of a SONIC DE system in two forensic labs*.

With the development of analytical microfluidic systems for any form of biochemical analysis, there are four fundamental 'X-ware' components - the '*chipware*', the *hardware*, the *software,* and the *'chemware'*. This applies equally to the SONIC system.

The 'chipware' is the microfluidic chip and, arguably the most important development in the overall system. The SONIC chip is simple in design (relative to other systems we have built) with six reservoirs connected to a single U-shaped channel that allows exposure of a flow stream to an acoustic piezo transducer in intimate contact with the channel. Fluid flow is controlled by a solenoid pumping system that is housed in the instrument and designed to avoid contact of fluid (sample, reagents) with the chip, thus avoiding cross-contamination.

The hardware involves the usual components - electronics, power supply, communication and slave boards, mechanical interfaces, fluidic and pneumatic components and optical (observation) electronics. The SONIC system possesses pumps fluids with 'off-the-shelf' syringe pump-driven hardware that easily allows independent control of flow between the six chip reservoirs. Critical to avoiding cross-contamination of the system, the syringe pumps pump air, not fluids, thus providing 'pneumatic' (as opposed to hydraulic with pumped liquids) control of fluid flow at all steps in the ADE process. Advances in delivery of the acoustic energy to the trapping node in the channel is facilitated by a small PZT that is minuscule in size relative to that utilized in earlier work [9], and cost-effective enough to be part of the single-use, disposable chip. Finally, a stand-alone cell phone camera has been built into the system to allow the user to visualize the trapping process in real time.

The software is laptop-driven (currently) and composed of a graphical user interface (GUI) for the user, and back-panel code that runs the system from sample input to male fraction collection. The GUI is designed to be user-friendly and allows the analyst in input critical sample information, see icon indicators that show the progress of the trapping process and a screen connected to the embedded camera that allows the user to observe the real-time trapping of sperm cells.

The 'chemware' is novel chemistry that involves the STR kit used for development of the SONIC system, as well as pre- and post-SONIC processing. There is a short (10 min) pre-SONIC step that

prepares the sample for loading into the chip in a form that optimizes sperm cell isolation via trapping. There is also a short (10 min) post--SONIC chemistry that, while not an absolute requirement for SONIC-based ADE, processes the isolated male fraction so that it contains the male DNA in a form that is ready for direct-to-PCR analysis. Finally, we utilize a 5- and 6-plex STR kit (developed in collaboration with Promega Corp) for the SONIC method development, primarily for budgetary purposes.

All combined, from a swab containing a mixture, the SONIC-based ADE process should yield a male fraction that is ready for PCR in 45-50 minutes. The effectiveness of this SONIC system was tested by delivering prototypes to two forensic labs - Kim Fiorucci-Meza at the Mesa Police Forensic Services (Arizona) and Dr. Cecelia Crouse at the Palm Beach County Sherriff's Office Crime Laboratory. The details and results of those evaluations are found in section 6 of this report.

2. INTRODUCTION

The backlog of samples awaiting forensic DNA analysis, both casework and convicted offender or arrestee, continues to be a significant social problem in the US [1,13]. Despite the technological capabilities of forensic laboratories, a recent summary from 153 labs reporting end-of-year casework backlogs, indicate that >150,000 samples await DNA analysis, with greater than half the labs reporting turnaround times of four months (or less) with some reaching as far as 9 months [2,12]. A sizable subset of the samples comprising the backlog involve sexual assault evidence (>27,500 of the ~150,000 samples), where roughly 40 % of these samples contain DNA evidence and require timely analysis for effective prosecution of suspected perpetrators [3,4 14,15]. There has been a realization that backlog reduction will not likely come from increasing the number of analysts, but from technology improvements that provide automation, decreased labor requirements and enhanced throughput.

With sexual assault evidence, obtaining a full, single source DNA profile is dependent on the efficient separation of sperm cells from female epithelial cells. Differential extraction (DE) is the most widely-used method of choice, employing proteinase K and an anionic detergent to elute material from a cotton swab collected from the victim, selectively lysing epithelial cells, leaving sperm cells from the perpetrator intact. DE involves numerous sample handling and centrifugation steps, leading to the potential for contamination and loss of valuable sample. In addition, it can suffer from a lack of separation specificity, with carryover from the female DNA sometimes seen in the male fraction, potentially making prosecution more challenging.

2A. CONVENTIONAL DIFFERENTIAL EXTRACTION

Forensic laboratories receive and process a wide variety of biological samples for short tandem repeat (STR) analysis. The majority of these samples are single-source samples, however, those which contain cells from multiple individuals yield mixed STR profiles - this is the case for sexual assault samples where vaginal swabs contain both perpetrator (male; sperm cells) and victim (female; epithelial cells), complicating direct identity linkage to the suspect(s).

The currently utilized differential extraction (DE) methodology, while effective, is labor-intensive, time-consuming, presents a higher risk for contamination, and infuses significant inefficiency to the overall workflow of forensic evidence processed in many laboratories. **Table 2-1** summarizes the details for some of the more commonly used DE processes. A well-developed robotic 96-well plate method provides a moderate-to-high throughput system using conventional DE methods and a

	Differential Extraction*	Laser Microdissection	Micro-manipulutation	Acoustic Differential Extraction**
Intital Setup Cost	$500-1000	$135-250K	$40-70K	$15-20K
Time Per Sample	at least 3 hours	depends on user ability & number of sperm cells desired	depends on user ability & number of sperm cells desired	45-60 minutes
Centrifugation Steps	15 (male fraction only)	None	None	None
Pipetting Steps	10 (male fraction only)	1	1-2	None
Analyst Skill Level (Training Required)	Moderate (moderate)	High (extensive)	High (extensive)	Low (minimal)

*From the QIAGEN QIAamp® DNA Investigator Handbook (p. 48)
**These values are esitmates for the deployable prototype and are subject to change.

Table 2-1. Comparison of ADE to other techniques used for the front-end processing of sexual assault samples.

commercialized system (i.e., Qiagen) that is ideal for running up to 96 samples in approximately >4 hours [3,14]). An alkaline modification procedure, for separation of male and female fractions, is compatible with the robotic 96-well plate method and reduces the sample preparation time by 2-fold [4,15]. This process, however, requires an extensive number (>16) of sample transfer steps, which significantly increases the susceptibility to error propagation and sample contamination [3,14]. Moreover, it requires multiple centrifugation steps for purification. As a result, the method is not actually fully-automated, as samples are manually transferred between the robotic system and the centrifuge. In addition, the sensitivity of the two methods is limited to >10 sperm cells/µL, therefore, recovery of sperm cells from a dilute forensically-relevant sample remains a fundamental challenge [3,14]. Finally, the cost of such robotic platforms can be prohibitive for many laboratories. Consequently, a need exists for a method that can eventually provide higher throughput with reduced analyst time, while avoiding generating a 'mixed STR profile' (perpetrator DNA contaminated with victim DNA).

With most forms of DE, differences in the stability of the cell membrane between these epithelial and sperm cell is exploited to separate male and female cells by differential extraction (DE). This method uses proteinase K (ProK) in combination with a detergent (sodium dodecyl sulfate; SDS) to elute cellular material from the cotton swab while selectively lysing the epithelial cells [15,16]. The sperm cells are pelleted by centrifugation, and the supernatant (containing DNA from the female) is removed. Following wash steps, a reducing agent (e.g., DTT) is used to lyse the sperm cells, and both 'fractions' progress through the remaining DNA preparation steps (extraction, PCR) to generate STR) profiles of the victim and the perpetrator via 5-color electrophoretic separation.

Although DE is the most common method for the analysis of sexual assault samples, the method is hampered by repeated sample handling (wash and centrifugation) steps, resulting in an overall process that is susceptible to contamination and is time-consuming [16,17]. Furthermore, if there is a large excess of epithelial cells present relative to a low number of sperm cells, DE may be ineffective at producing a male fraction which contains only male DNA, resulting in the preferential amplification of female DNA during PCR, obscuring the male STR profile [17,18]. As a result, significant effort is being exerted by a number of groups to improve the processing of sexual assault samples with DE for enhanced recovery of sperm cells and/or improving the purity of the male fraction and reducing analysis time.

To enhance the recovery of the male DNA, Tereba, *et al.* [18,19] developed the Differex™ System, for efficient separation of male and female DNA using a *Separation Solution* that is less dense than sperm cells, but more dense than water. During centrifugation, the sperm cells are passed through the *Separation Solution* and form a pellet at the bottom of the tube, while DNA from lysed cells remains in the aqueous layer. Although this method does provide separation of male and female DNA, there is still some carryover of female DNA into the male fraction.

Alternative approaches opt to NOT separate the epithelial cell lysate from the sperm cells, and simply 'remove' female DNA by degrading it with DNase I [19,20 20,21]. Following epithelia cell lysis, degradation with female DNA by DNase I and inactivation of the DNase, a reducing agent is added to lyse the sperm cells, and both the female and male fractions are carried through the

remaining DNA analysis steps. However, if female DNA was not adequately degraded, a mixed STR profile results from the sperm cell lysate.

Our laboratory has focused on the enhancement of sperm cell elution and recovery by modifying the chemistry of the DE process. This has included: a) the use of cellulase-based enzyme mixtures to dissolve the fibrous cellulose network of the cotton swab [21,22], b) the utilization of different detergents as elution agents to enhance sperm cell recovery [22,23] and, c) leveraging *b)*, a one-step cell elution and preferential lysis method to recover up to 85% of sperm cells from a cotton swab matrix while comprehensively lysing the epithelial cells in a 30 min incubation [13,10]. With this last method, a portion of a cotton swab containing both epithelial and sperm cells was added to the one-step buffer (10 mM Tris; 10 mM MES) with 1 % SDS and ProK. The majority of cellular material is eluted and epithelial cells are selectively lysed during the 30 min incubation (42°C), reducing sample prep time and maximizing recovery of intact sperm cells.

2B. MICROSCALE SPERM CELL ISOLATION METHODS

Along with the chemical techniques described in the previous section, there are physical manipulation methods for the separation and isolation of sperm cells. Precise isolation and gathering of sperm cells has been achieved using micropipettes and microspheres. Petit, *et al*. demonstrated isolation of single sperm cells that, after whole genome amplification, allowed for the generation of partial profiles [23 24]. More recently, Schneider, *et al.* demonstrated isolation by 'picking' sperm cells from a mixed sample using microspheres. Sperm cells adhere to the microspheres, manipulated by custom grippers, allowing for careful movement of the spheres to only collect sperm cells. After collection, full STR profiles were obtained from as few as 20 sperm cells with no evidence of epithelial cell DNA carryover [24 25]. In addition, laser microdissection [25 25a] has become a popular technique for isolating sperm cells from microscope slides. Generally, mixed samples are smeared and dried on a polyethylene napthalate (PEN) microscope slide. Sperm cells are laser-cut from the slide and a defocused laser pulse catapults the cells into a waiting aliquot of buffer directly above the location of the cell. STR profiles obtained from sperm cells isolated by this method also show little evidence of epithelial cell DNA carryover. However, in general, these methods require costly instrumentation and/or a highly trained analyst to select which and how many sperm cells are to be captured, and thus, are not amenable to high throughput analysis.

Fluid flow and chemical/biochemical reactions in the microfluidic regime offer a number of characteristics that address drawbacks of the standard DE method. Immediate benefits include reducing: 1) sample and reagent volumes, 2) contamination in a 'closed system', 3) the number of sample transfer steps and, 4) potential sample loss [5-11 1-3,6-8]. This is particularly the case if integrated with more than one analysis step, yielding the possibility for faster analysis times [29 26]. Cell manipulation and sorting techniques have been demonstrated on microfluidic devices [30 27] using techniques such as dielectrophoresis [31,32 28,29] and fluorescent-activated cell sorting [33,34 30,31]. Dielectrophoresis utilizes a non-uniform electric field to separate cells based on differences in dielectric properties [35 32] and has been used to separate live and dead cells [36 32b] or cells in different cell-cycle phases [37 33]. A drawback to dielectrophoresis, however, is that

cells may adhere to the microchannel walls at the trapping site, decreasing the effectiveness of the separation method [38 33b]. In contrast, fluorescent-activated cell sorting (FACS) uses a fluorophore-labeled antibody which interacts with the target cell type. A laser is used to excite the fluorophore and the flow is switched to direct the targeted cells to a separate reservoir [39 34]. This method has been successful in separating *E. coli* cells expressing green fluorescent protein from non-fluorescent *E. coli* cells [40 35]. However, microfluidic FACS (µFACS) uses a multilayer microchip structure [41 36], requiring numerous fabrication steps (hence, expense) and an increased risk of clogging in the microchannels [39 34].

In a different approach, we demonstrated successful separation of sperm and epithelial cells on a microdevice by exploiting differences in their physical properties with focus on two approaches. The first allowed epithelial cells, with their inherent larger size and higher density, to settle and adhere to the bottom of an inlet reservoir while buffer flowed through the device mobilized sperm cells towards the outlet reservoir [42 37]. This method required adequate time for the epithelial cells to settle, assumed minimal lysis of the epithelial cells during the process, and had a relatively poor volume throughput [42 37]. A second method developed in our laboratory exploited an acoustic energy to trap sperm cells from a cell lysate. While several cell sorting techniques have been demonstrated on microfluidic devices, the use of acoustic trapping was particularly attractive because of its simplicity - low power acoustic energy applied to a fluid-filled microchannel generating a standing acoustic wave that creates low pressure zones capable of trapping particles in a size-selective manner [12 9]. The applicability of this to DE led us to explore ADE as a potential method for rapidly extracting sperm cells out of lysate from a swab containing epithelial and sperm cells in the form of an NIJ 'proof-of-concept (POC)' grant for ADE (2006-DN-BX-K021),

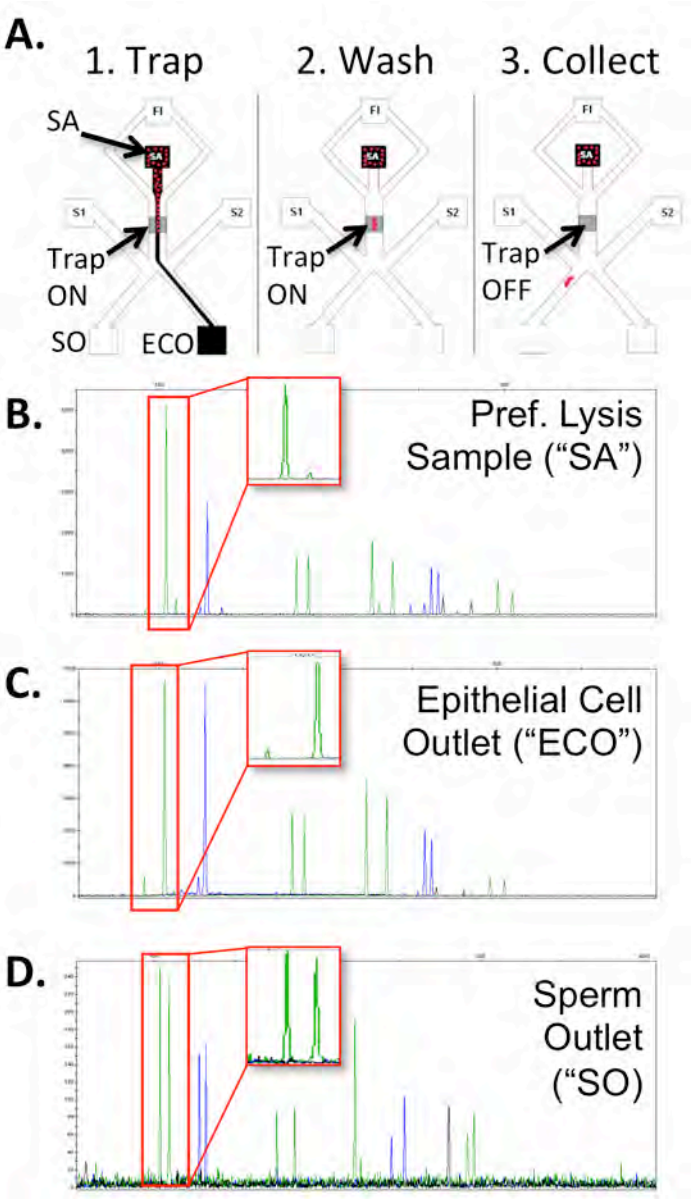


Figure 2-1. Proof of principle for ADE from Norris *et al.* (12). (A) The microdevice operation is shown in 3 steps: 1. the transducer is actuated to retain sperm cells as the epithelial cell lysate is flowed through the device; 2. cells are retained in the trap as clean buffer is infused; and 3. the transducer is turned off and flow is directed to the sperm cell outlet (SO) to collect trap contents. (B) Cofiler STR profile of the original sample (SA), with the amelogenenin peak enlarged (inset). (C)STR profile for clarified epithelial cell lysate. (D) STR

where we developed a breadboard and demonstrated the effective trapping of sperm cells in the presence of lysed epithelial cells. Collection of the trapped sperm cells occurred by simply terminating voltage to the transducer, and switching the flow (laminar flow valving) to direct the sperm cells to a separate outlet reservoir. This is summarized in **Figure 2-1** which shows the results from that original work (using what we now consider to be a primitive fluidic architecture), where the processing of a mixed sample resulted in a male fraction with a purity as high as 92%, a 4-fold increase over the original sample [12 9].

2C. ACOUSTIC CELL TRAPPING THEORY

Acoustic cell manipulation techniques employ the use of standing acoustic waves generated within a resonant cavity to move or capture particles and/or cells as they pass through. Acoustic standing waves have been extensively used on the macroscale to aggregate and sediment cells, such as yeast [43,44 38,39] and red blood cells [45,46 40,41], allowing collection at the bottom of a stagnant chamber. While an in-depth understanding of the physics of acoustic trapping is not essential to this proposal, suffice to say that cell movement is dictated by the primary axial radiation force (F_r) acting on it in an acoustic standing wave described by:

$$F_r = \left(\frac{\pi P_0^{\,2} V_c \beta_w}{2\lambda} \right) \phi \sin(2kx)$$

where P_0 is the applied acoustic pressure amplitude, V_c is the volume of the particle (i.e., size), λ is the acoustic standing wavelength, k is the wavenumber defined by $2\pi/\lambda$, x is the distance from a pressure node, ρ_c and β_c are the density and compressibility of a particle, respectively, in a fluid with density ρ_w and compressibility β_w, and ϕ is the acoustic contrast factor and is given by:

$$\phi = \frac{5\rho_c - 2\rho_w}{2\rho_c + \rho_w} - \frac{\beta_c}{\beta_w}$$

This axial force acts in the direction of propagation of the acoustic wave field and is responsible for the movement of the particles and/or cells to the nodes or anti-nodes of the wave (see **Fig. 2-2B**). As evident from the equations above, the axial radiation force (i.e., the trapping force, F_r) is dependent on a number of particle/cell factors, including the compressibility, density, size and shape of the particle as well as the particle size, where a decrease in size results in a decrease of the acoustic force. In addition to the F_r, there are also secondary acoustic forces acting upon a particle that result from the scattering of acoustic waves by other particles, causing particles to be attracted to or repulsed from one another. Although these interparticle forces are much weaker than the F_r, they are significant when attempting to aggregate and sediment cells [47,42]. Also, note the dependence of the acoustic factor on the density (ρ_w) and compressibility (β_w) of the fluid surrounding the cells. This indicates that strict attention be paid to the solutions/buffers used during a trapping event.

2D. APPLICATIONS OF CELL MANIPULATION VIA ULTRASONIC STANDING WAVES

There are two main operational modes for cell handling in microfluidic acoustic wave resonators: focusing and trapping [46-51 41-46]. Acoustic forces may be used in a flow-through technique called free flow acoustophoresis, where an ultrasonic standing wave is established along the entire length of a microchannel with parallel walls. This mode, carried out at lower acoustic frequencies (~2 MHz), does not trap but, instead, focuses particles to the center or to the walls of microchannel. While effective for resolving bacteria from viruses [52,47], separating blood cells from fat globules [53,48], and achieving buffer exchange [54,49], it does not enhance cell concentration per unit volume, hence, is not exploited in this proposal. In contrast, acoustic trapping occurs at higher acoustic frequencies (~10 MHz) and utilizes a standing wave that is established in very localized zones. Thus, as the cell suspension is infused through the microdevice, particles above a certain size (dictated by the interplay between acoustic and viscous drag forces as describe above) will become trapped at the pressure node(s) within the channel. Acoustic trapping has been used to culture yeast cells [51,46] and again, based on development with past NIJ funding, used to separate sperm cells from buccal swab lysate using the ADE method described above [12,9]. We have selected the

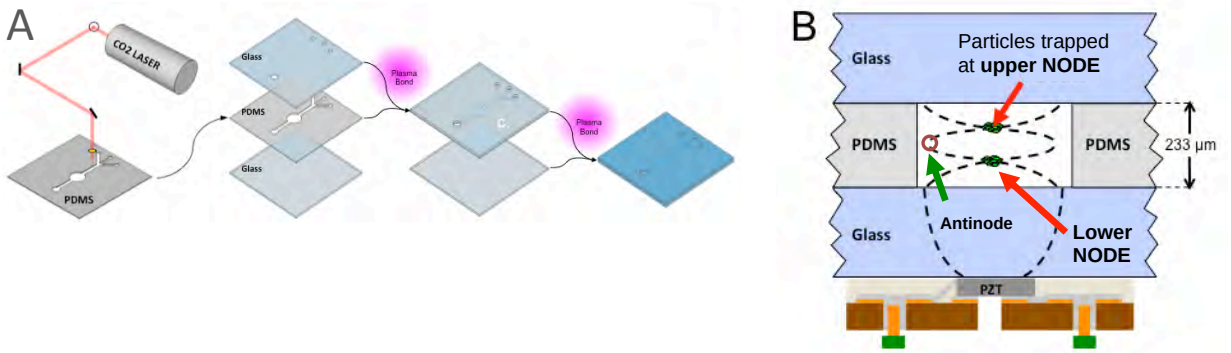


Figure 2-2. Fabrication of a glass-PDMS-glass microdevice. (A) Laser ablation and assembly of the layers to generate a microfluidic device with an acceptable resonator cavity. (B) Side-view of the resonator cavity with a standing acoustic wave generated by a piezo (PZT).

trapping mode because it can accept a wider range of input cell concentrations while producing the same output concentration of cells (as determined by trap contents and trap to collection tube dead volume). This is attractive for male STR typing, as the output DNA concentration may be semi-quantitatively judged from the number of trapped cells.

The proposed developments will utilize a low-cost, easy-to-fabricate microfluidic acoustic trapping device that is fully-enclosed and disposable (avoid cross-contamination) made from glass and a flexible silicone (polydimethylsiloxane; PDMS). We have refined the fabrication of these glass-PDMS-glass (GPG) hybrid devices [68,71-73 50,51-53] using a laser ablation system to create the microfluidic structures in the silicone layer, sandwiched by glass layers provide the features that allow for access to the microfluidic structures, as well as the ceiling and floor of the resonator cavity for acoustic standing wave generation (**Fig. 2-2**). These devices are capable of trapping cells with greater throughput and trapping efficiency relative to our previous ADE work [12,9]. The GPG devices will feature a microchannel architecture that allows for faster sample flow rates, and, as a

result, accommodation of samples up to milliliter volumes, often representative of samples that a forensic laboratory. The implementation of bead-assisted trapping will allow for the capture of sperm cells from samples containing trace number of sperm cells (<1 cell/µL). In addition, the geometrical arrangement of multiple transducers (up to 4) will be evaluated to determine the optimal geometry of the transducers needed to provide high trapping efficiencies (≥60 %). Finally, since ADE is dependent on efficient recovery of intact sperm cells from the cotton swab and comprehensive lysis of epithelial cells, the recovery of sperm cells and the preferential lysis of epithelial cells using an enzyme-based DNA preparation method will be evaluated. In addition, a modified enzyme-based DNA preparation technique employing TCEP as the reducing agent, will be used to provide on-chip lysis of the sperm cells after trapping and washing, minimizing sample loss to the walls upon release of the cell bolus.

2E. FURTHER DEVELOPMENT IN THE INTERVENING TIME BETWEEN NIJ AWARDS

With funding obtained after the previous 2006 NIJ funding period ended, we made a number of significant advances beyond the proof-of-principle reported in the Norris *et al.* paper [12,9]. They can be summarized as: (i) obtaining the first evidence of trapping sperm cells at flow rates as high as 60 µL/min, (ii) obtaining efficient trapping of cells in diluted semen at concentrations <1 cell/µL, (iii) creating smart-engineered circuit board-supported transducers, and (iv) creating a primitive GUI program for remote control of the ADE process. This provides the framework upon which we carry out the final R&D required to generate prototype ADE devices and instrument for testing in forensic labs, preferably from commercial-of-the-shelf (COTS) components.

In the intervening time between the ending of the 2006 and the award beginning in 2013, progress continued on the acoustic DE system. That effort was directed at improving the acoustics-microchip interface, acoustic energy delivery to the trap zone, sample delivery methods and male/female fraction collection. This work was primarily funded through a one year subcontract with Lockheed Martin. This placed us in a position where we had adequate confidence on the ability of acoustics to provide physical separation of the male and female fractions, supported by preliminary data, to propose a second round of NIJ funding that would allow for us to create three prototype devices that would allow us to: 1) define the ultimate parameters for efficient sperm cells capture on mock samples over a rang of female:male cell ratios, and 2) place two instruments in reputable forensic laboratories for testing on bona fide forensic samples.

We experienced some major challenges in terms of obtaining efficient trapping and physical separation of the male and female fractions when the sperm cells were in a over abundance of epithelial cells. The first year of this to your effort involved optimizing trapping efficiency under a variety of conditions and in the presence of other contaminating cells. By the beginning of Q2 in the second year, we had overcome the challenges associated with the 'chemistry' and physical trapping of the male fraction. It was at this point that we begin the design and engineering of an instrument that could be delivered to other labs for testing – in other words, an instrument that wasn't simply built on a solid aluminum optical breadboard with screw-holes that allowed for the mounting components in three dimensions. This effort consumed the latter part of the funding period.

3. METHODS

3A. MOCK SAMPLES

Information Gathering

Due to the sensitive and timely nature of sexual assault kits, *bona fide* forensic samples were not used during the development of this method. Rather, mock samples were generated in various states, using the best information available to mimic realistic scenarios. We spoke with forensics experts in multiple state and federal labs (6 analysts in 4 different forensic labs; identity held as confidential) to ascertain what a "typical" swab sample would be, and found that the definition of typical swab was very lab-dependent. However, the responses did provide information regarding average sample composition, sperm cells recovery efficiency, and general ratios of female:male cells present on a swab. The most important information gleaned from these responses was that a typical sexual assault kit sample (cutting of 1/2 of a cotton swab) will contain anywhere from 300 sperm cells up to 17,000 sperm cells, at a female:male cell ratio of 10:1 up to 50:1. Based upon this information, mock samples were formulated within the relevant forensic range for total cell number and ratio of female:male cells.

Acquisition of Swabs

Cheek swabs were collected in a manner consistent with an IRB established for this project, and reference STR profiles obtained for comparison purposes. Post-coital vaginal swabs were obtained through a collaboration with Dr. Kathryn Laughon of the UVA Medical Center (under her IRB). All swabs were de-identified, with sample tracking carried out using the donor ID number, swab type, and cell yields; examples are displayed in **Table 3-1**.

It should be noted that we trialed two different kinds of swabs - the more traditional cotton swab used extensively, and a flocked swab from *Copan Italia spa*. While the flocked swab provided more cells (by more than 2-fold), traditional cotton swabs are used in Virginia criminal forensic labs. For this reason, cotton swabs were primarily used to generate mock samples.

Donor ID	Cell Type	Swab Type	Cell Count (per uL)
K15J	Cheek	Cotton	1025
A08T	Cheek	Cotton	222
003	Vaginal	Cotton	306
004	Vaginal	Cotton	744
004	Vaginal	Cotton	2167
010	Vaginal	Cotton	546
006	Vaginal	Cotton	588
013	Vaginal	Cotton	405
015	Vaginal	Cotton	189
012	Vaginal	Flocked	1428
012	Vaginal	Cotton	656
017	Vaginal	Cotton	738

Table 3-1. Cataloged sample submission information.

Acquisition of Semen

A listing through UVA Clinical Trials resulted in semen donation from anonymous males. The cell count from this donor was determined to be 160,000 sperm cells per microliter in one batch, and 91,000 sperm cells per microliter in the second

collection. The semen was split into 50 µL aliquots, and stored at 4°C between uses. Prior to generating mock samples, aliquots of neat semen were diluted to a final concentration of 2,000 cells per microliter. Direct comparison between samples showed that sperm cell viability diminished rapidly once diluted (compare profiles of same dilution, 1 week apart). As a result of our sample degradation, a new aliquot of neat semen was freshly diluted for each trapping experiment.

Liquid vs Dried Samples

The presence of sperm cells in the post-coital swab samples obtained from Dr. Kathryn Laughon at the UVA Medical Center was unknown. Unfortunately, no sperm cells were present on any post-coital swabs, forcing us to utilize these to create mock samples by spiking with semen (that had been assessed in terms of sperm count). In some cases, sperm cells were spiked directly onto the swab and allowed to dry overnight - we refer to these as "dried" samples. These more closely mimicked a *bona fide* sexual assault kit swab, with the advantage of knowing the number of sperm cells added. With some knowledge of the epithelial cell count (hemocytometer), the female:male cell ratio was could be estimated.

Samples were also prepared by reconstituting the swabs in water; these were referred to as "liquid" samples. Again, knowing the number of sperm cells added to a known amount of epithelial cells, a more accurate female:male cell ratio for a sample could be established.

3B. STR PROFILES FOR EVALUATION ADE

Use of a Custom 6-plex STR Kit

A custom 6-plex kit (courtesy of *Promega, Corp*) was used for this work, primarily because we had an ample supply of this particular chemistry kit in the lab. It was our opinion that, given this involved development of a 'first-of-its-kind' instrument and methodology that would require ~$30K if the currently used multiplex reagents (e.g., GlobalFiler) were used. Given the limitations of budget, we opted for the lower-plex system. The loci involved were amelogenin, (AMEL) D18S51 (D18), D2S1338 (D2), D21S11 (D21), D8S1179 (D8) and D12S391 (D12). The 6-plex primer mix was used with a proprietary master mix based on the PowerPlex® Fusion System, but enhanced position the SONIC chip for potential on-chip PCR. The chemistry exploited composed of three dyes - two standard dyes (FAM, JOE) and

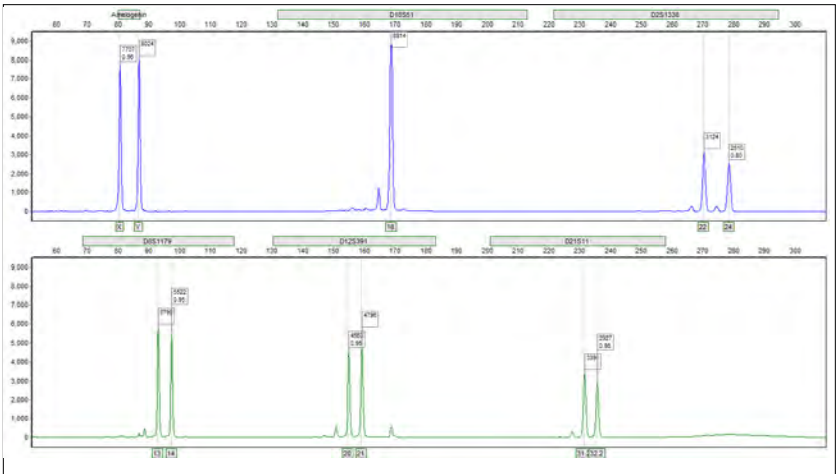


Figure 3-1. STR profile of extracted semen from donor 5YOB. Strong peak

one energy transfer dye (ET-CRX). The amplification was performed in 49 minutes using an Eppendorf Mastercylcer. The thermocycling parameters were: 30 cycles of 94°C for 10 seconds, 60°C for 60 seconds, and a final extension at 60°C for 5 minutes.

The products from all PCR amplifications were analyzed using an ABI 3130 Genetic analyzer (*Life Technologies, Carlsbad, CA, USA*). The injection and separation conditions were 15 KV for 5 seconds and 15 KV for 1680 seconds, respectively. Genemarker HID software (Softgenetics LLC, State College, PA, USA) was used to analyze the profiles with a binning palette that was manually created to fit the new 6-plex. An allelic ladder was provided by Promega to create this new binning palette containing the 6 markers. An example of the profile generated with this STR kit is seen in **Figure 3-1**. As will be described in a subsequent section, the presence of primers for D18S51 wreaked havoc on the overall efficiency of the PCR, hence, for some experimental sets this has been removed.

3C. SAMPLE PROCESSING

Sperm Cells on Mock Dry Swabs

For mock samples that had sperm cells dried directly onto the swabs, a cutting was taken to obtain cells. Using a disinfected surface and fresh razor blade, the cotton swab was sliced vertically on each side, allowing for half of the swab to be separated. This swab cutting was then suspended in liquid for cell lysis, as detailed below.

Sperm Cells in Mock Liquid Samples

The SONIC system is designed to accommodate anywhere from 30 to 120 µL of sample volume. Its size is commensurate with future developments where we plan to perform all pre-SONIC sample preparation on-chip. In these studies described here, samples were prepared at an initial volume of 60 µL with differing numbers of male and female cells - this ranged from 1,000 - 50,000 female epithelial cells, and 200 - 2,000 sperm cells. The volume of cells added to create each sample varied with the donor, dependent upon the concentration of cells recovered from the cheek or vaginal swab.

Preferential Lysis

The first step in any differential extraction is to lyse in solution all of the female cells which, in most cases, is dominated by epithelial cells. Conventional differential extraction utilizes a detergent concentration that lyses epithelial cells while leaving perm cells intact. Recall (section 2C) that the acoustic contrast factor and, hence, trapping efficiency, was influenced by the properties of the buffer. As a result of this, we utilized a unique chemistry for epithelial cell lysis. This involved *prep*GEM enzyme and ZyGEM Blue Buffer (ZyGEM NZ Ltd), incubated at 75°C for 15 minutes to rupture the epithelial cells, and 95°C for 5 minutes to heat-kill the enzyme. Following this, sarkosyl was added so that the final concentration was at 0.6%; this served to solubilize cellular membrane debris, which could adversely affect trapping of sperm cells. With this initial lysis step completed, the sample was ready for trapping on the SONIC instrument. If the lysis was performed on a swab

cutting rather than a liquid sample, the cutting was removed after this lysis step. For reasons that will be clear later, 6 µm polystyrene spheres 1 were added to samples, in order to initiate aggregation of sperm cells in the trap site, something that is critical when a low number of sperm cells are present.

Materials for Chip Fabrication

The bottom glass layer of the acoustic resonator is a piece of uncut 180 µm thick borosilicate glass, and the top glass layer is a piece of 180 µm thick borosilicate glass that is cut through by laser ablation to make 6 access holes for the 6 reservoirs on top of the GPG assembly. The PDMS layer is made from a 280 µm thick commercially available PDMS film and all the channels are generated by laser ablation. The reservoir layer is made from a 1/8 inch PMMA sheet that has been pre-adhered to a layer of double side pressure sensitive adhesive (PSA) on one surface and cut through by laser ablation.

Instrument Operation

The SONIC instrument (**Fig. 3-2**) must be powered up 30 seconds before the computer, to allow booting of all necessary drivers. After both the instrument and laptop are powered up, the LabView application can be operated for sample trapping. The first step for the user is to initialize the syringe pumps, which draw back to a volume of 100 µL. It is imperative that this step be completed prior to loading the chip onto the manifold, or else sample could be drawn into the pumps and system contamination could occur. After initialization the chip is loaded onto the instrument, and sample processing begins. The next step is a crucial one and involves sweeping through multiple acoustic frequencies for each chip. The 'frequency sweep' applies a range of frequencies to the piezo, while 6 µm fluorescent beads are flowed through the trap site. After passing through the trapping site, the test bead solution is diverted to the waste chamber, reservoir 6. Custom software will have identified optimal frequency from 8 distinct trapping frequencies. That frequency is chosen for sample trapping throughout the rest of the run. Next the piezo is activated at the optimal frequency, and the sample is flowed through the trapping site at a rate of 45 µL/minute. Sperm cells are trapped by the standing acoustic wave (**Fig. 3-3**), while free DNA and cellular debris are washed downstream to the non-sperm chamber (reservoir 5). The trapped sperm pellet is then washed with buffer to remove any remaining debris. Finally the piezo is deactivated, and the pellet is eluted to reservoir 4, referred to as the sperm fraction. In total the system operation takes 7 minutes per sample.

Figure 3-2. The SONIC system for acoustic differential extraction of sperm cells.

Sample Recovery

At the conclusion of the run, the sperm pellet has eluted in final volume of 30 uL. Reservoirs 4, 5, and 6 of every chip are pre-treated with Sigmacote, a siliconizing reagent which prevents back-flow of sample into the microfluidic channels. The pellet is easily pipetted off of the chip, and the sperm cells are ready for extraction.

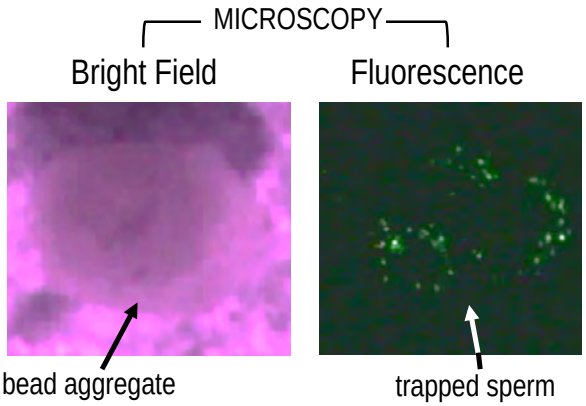


Figure 3-3. Visualization of trapped 6 µm beads and trapped cyto11-labeled sperm cells. via both bright field and fluorescence microscopy.

DNase Treatment

A small amount of non-sperm DNA may remain in the sperm fraction, so this 30 µL sample is treated with DNase 1 enzyme and DNase buffer (ZyGEM NZ Ltd) and incubated at 37°C for 5 minutes, followed by 75°C for 5 minutes. By performing the DNase step after SONIC separation, an STR profile from both the sperm and non-sperm fractions can be obtained, allowing for direct comparison to the initial mixed sample.

Male DNA Extraction

Sperm cells are lysed using Accrosolv, *prep*Gem, and Red+ buffer (ZyGEM NZ Ltd) by incubating at 52°C for 5 minutes, 75°C for 3 minutes, and 95°C for 3 minutes.

DNA Amplification

Promega reagents were used for all PCR amplification, including the PP18 and Fusion kits, as well as custom 6-plex and 5-plex primer sets. An initial 96°C denaturation step was followed by 30 cycles of 94°C for 10 seconds and 60°C for 60 seconds, before concluding with a 60°C hold for 5 minutes.

DNA Separation

Early separations were performed with an ABI 310 instrument, before upgrading to a 16-capillary ABI 3130. Many factors of the post-SONIC processing, including PCR reagents, ABI instrument maintenance, and capillary performance varied significantly over time, which accounts for some of the variation in peak heights observed throughout the data.

STR Analysis

All samples contained ILS 500 for determination of peaks, and were analyzed using GeneMapper software version 2.8.2. A control male profile from a semen donor was compared to the profiles from the sperm and non-sperm fractions after every SONIC run, in order to determine if an isolated male profile was obtained. An exemplary male profile was given in **Figure 3-1**.

4. RESULTS

The SONIC (**S**EX **O**FFENDER **N**ODAL **I**SOLATION OF **C**ELLS) System is an unprecedented analytical system capable of trapping cells and particles that have a particular size, shape, density and compressibility (see equation on page 9). This attempts to exploit the growing interest in microscale ultrasound technology for augmenting, complimenting or supplanting centrifugally-driven separations [39-46,54]. DE is an excellent example of a process desperately in need of new technology to supplant pipetting and centrifugation. While there are nuance differences in the methodologies employed and validated by labs nationwide (see **Table 4-1)**, there seems to be succinct agreement that there is a defined element of skill and subjectivity in the process and, perhaps, most importantly, it can be time-consuming. The forensic community needs a DE process that is objective, automated and rapid - the acoustic DE approach we describe here presents that possibility. The process we describe, which a combination of off-chip and on-chip processes, is encouraging, as it should process a swab cutting to yield a PCR-ready male fraction in less than 1 hour. However, this represents the product of a Phase-2 effort in what we believe is a three Phase process. Defining the limitations of the SONIC system is ongoing and, ultimately, this will be finalized by placement into two forensic labs in Q1 2017. We understand that a system that processes a single sample is of limited use to many labs, however, those developing analytical technology know that you demonstrate proof-of-principle with a singe sample system before mutliplexing chip or the hardware. As discussed in *Discussion and Concluding Remarks* (section 5), the instrument has been designed to be low cost and, should it undergo 'productization', with a low rate production (e.g., 100 instruments) retail for less $20K.

	Differential Extraction*	Laser Microdissection	Micro-maniplutation	Acoustic Differential Extraction**
Intital Setup Cost	$500-1000	$135-250K depends on user ability & number of sperm cells desired	$40-70K depends on user ability & number of sperm cells desired	$15-20K
Time Per Sample	at least 3 hours			45-60 minutes
Centrifugation Steps	15 (male fraction only)	None	None	None
Pipetting Steps	10 (male fraction only)	1	1-2	None
Analyst Skill Level (Training Required)	Moderate (moderate)	High (extensive)	High (extensive)	Low (minimal)

*From the QIAGEN QIAamp® DNA Investigator Handbook (p. 48)
**These values are esitmates for the deployable prototype and are subject to change.

Table 4-1. Comparison of ADE to other techniques used for the front-end processing of sexual assault samples.

4A. THE SONIC CHIP

SONIC Microfluidic Chip Design

The architecture of the SONIC chip, as given in **Figure 4-1A,** is a multi-layer, multi-material hybrid microdevice that features two major domains in functionality. The first domain is the 'resonator domain' where ultrasonic standing waves are established between two glass surfaces spaced by a PDMS layer with specific distance that matches the conditions for ultrasonic standing waves. The second domain is a 'fluidic interface' domain where the liquid reagents are initially loaded, and then driven and collected by external syringe pumping/valving operations. The PDMS layer at the top of the interfacing domain, when pressed against the o-ring in the locking unit, ensures the airtight

connection with the external pressure sources and allows for tolerance of the different chip height within our manufacturing ranges (**Fig. 4-1B**). The channels are 1.0 mm in width, and approximately 280 µm in height. The piezo transducer (PZT) applies the standing acoustic wave, and is positioned over the trap site. Reservoir 1 is first treated with 10 µL of priming solution (5:1:1 ethanol, glycerol, and water) in order to wet the channel and reduce dead volume on the chip. 110 µL of test bead solution (1:500 fluorescent beads and water) is then added to reservoir 1. This test bead solution is used to identify the unique optimal trapping frequency for each chip, which will vary based on channel height and layer thickness (see section 4A.4). The relationship between channel height and optimal trapping frequency is shown in **Figure 4-2**. The current protocol involves the addition of 65 µL of sample to reservoir 2, and 43 µL of wash buffer to reservoir 3.

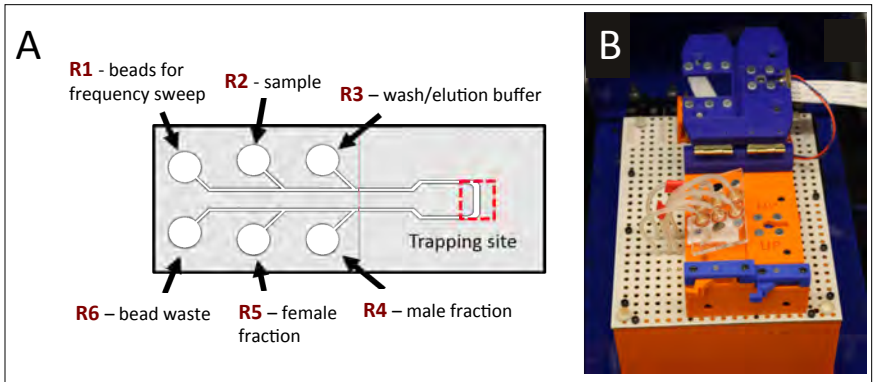


Figure 4-1. Microfluidic chip and SONIC instrument. (A) SONIC microfluidic chip composed of glass, PDMS, PMMA, and double-sided adhesive. (B) SONIC instrument manifold. Chip is mounted beneath rubber o-rings, which provide connection to syringe pumps and valving.

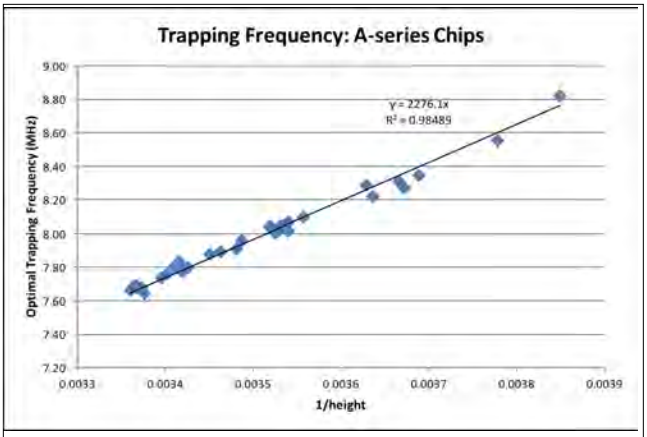


Figure 4-2. Relationship between channel height and trapping frequency. A linear relationship is observed between the inner channel height of the trapping site and the optimal trapping frequency of the piezo. This relationship is used to

Microfluidic Chip Fabrication

Assembly of the Fabricated Parts

The PDMS layer is solvent-bonded with the top glass layer by ethanol-assisted alignment and evaporation in vacuum. After bubble removal between the PDMS layer and the glass top layer, the PDMS-glass pre-bonded assembly is plasma bonded with the bottom glass layer to form a tight, irreversibly bonded GPG assembly. The non-PSA coated side of the PMMA reservoir layer is bonded with a PDMS film that is PSA coated and 1.6 mm thick, and then acu-punched through the PMMA reservoir holes to cut through the PDMS. Then PMMA-PDMS reservoir assembly is adhered to the GPG assembly through the double side PSA on the non-PDMS attached side of the PMMA layer. Fabrication is completed by attaching a piezo transducer to the bottom of the resonator chamber using epoxy. **Figure 4-3** provides a high level schematic of the process.

Surface Treatment

The exposed glass in the access holes in the three downstream reservoirs (R4, R5, R6) is treated with Sigmacote® to provide a hydrophobic barrier to contain the solution to these reservoirs. As a result of this simple treatment, after fluid is fully discharged(displaced) by air from the channel, fluid remains in the reservoir and does not experience backflow into the channel.

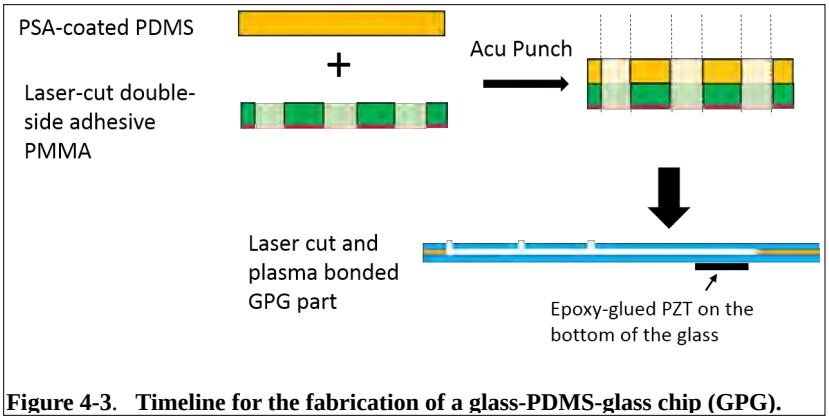


Figure 4-3. Timeline for the fabrication of a glass-PDMS-glass chip (GPG).

Microfluidic Chip QC

Accurate Fluid fLow Control

As described in more detail in the hardware section, fluidic flow is accomplished with commercial-off-the-shelf syringe pumps that pneumatically-drive fluidic movement within the chip architecture. Pneumatic (not hydraulic) is a logical approach when attempting isolate the hardware from contact with fluid in the chip. While the chips are single-use disposable, residual fluid within the flow control system presents a potential for cross-contamination. As shown in **Figure 4-4**, The flow direction is selectively switched between reservoirs by start/stop commands to the syringe and/or opening/closing the solenoid-controlled downstream valves. To ensure a reproducible flow is

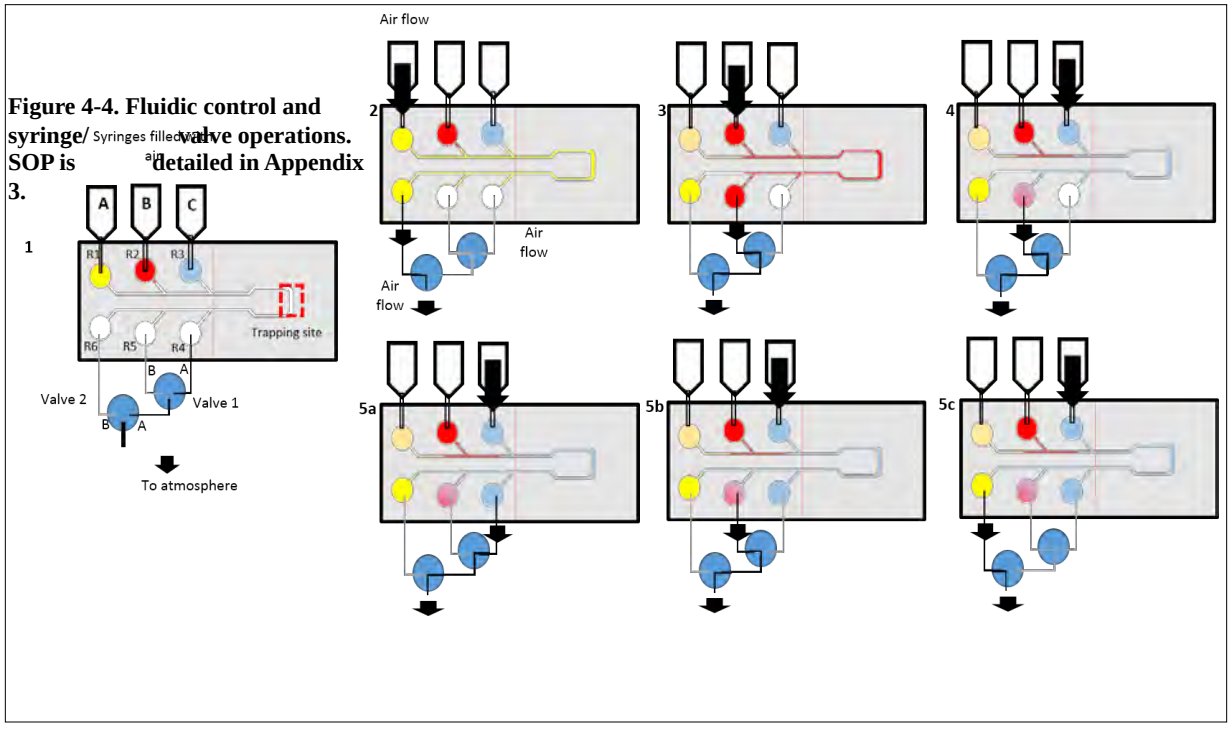


Figure 4-4. Fluidic control and syringe/valve operations. Syringes filled with air. **SOP is detailed in Appendix 3.**

established from run to run and in the appropriate direction, an air-tight seal between the chip and the chip interface module (CIM) is essential. In early stage experiments, dye solutions were used to assure flow control, direction and final destination. The correct movement of dye solutions was established through analysis of the solution in R4, R5 and R6 at the end of the fluid pumping protocol. Spectroscopic analysis of the resultant solutions is a sensitive method for determining whether any cross-contamination or other dead volume carry-over in the channel has occurred. None was observed. **Figure 4-5** shows the result of the 'demo' fluidic run, using yellow dye, red dye and H_2O to represent the bead test solution, the sample solution and wash/elution buffer, respectively. Repeat analysis of this nature demonstrated that flow control, direction and final destination of the R4, R5 and R6 solutions could be manipulated appropriately with minimal reservoir to reservoir cross-contamination.

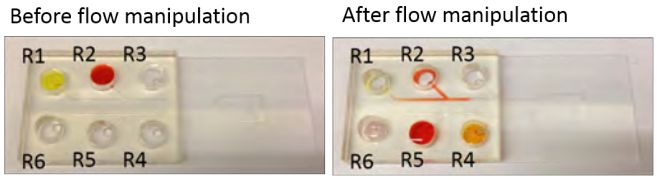


Figure 4-5. Initial dye experiments with the SONIC chip to assure

Importance of Piezo Transducer (PZT) Frequency

PZTs that resonate at a range of frequencies from single kHz to several MHz can be purchased from a variety of commercial suppliers. Both the resonance frequency range of the piezo and the the height of the resonator channel (explained in depth below) factor into how many nodes exist in the trap zone. We initially utilized 5 MHz PZTs, then upgraded to 7 MHz which was much more effective.

The 5 MHz PZT interfaced with resonator channel height around 280 nm showed the best trapping efficiency at a resonance frequency of 5.4 MHz; however, this only provided a single nodal plane, i.e., a single trap site. Each trapping site has a specific cell capacity which, in our system, was several thousand cells, beyond which cells are not trapped, ultimately effecting trapping efficiency. This is not problematic with samples having a large number of sperm cells, but could be with low sperm cell number samples. A simple solution is to increase capacity by creating more nodal planes in the same resonator cavity.

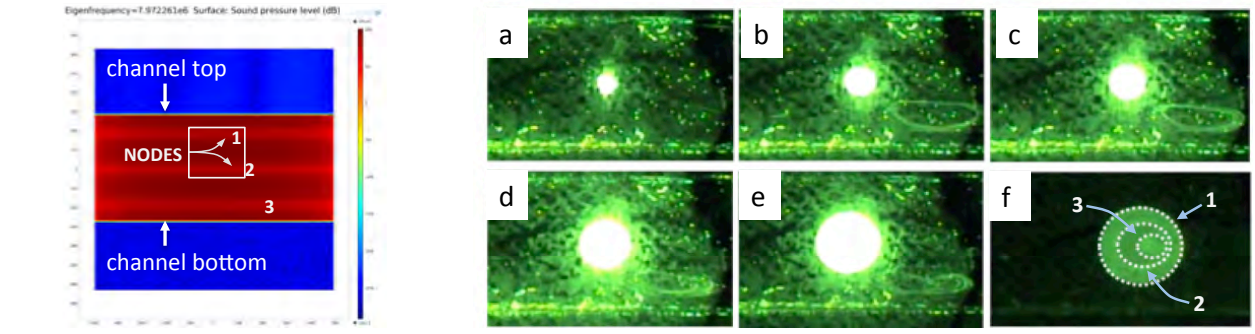


Figure 4-6. Defining optimal trapping capacity with increasing number of nodes. (A) Figure 1. Simulated trapping nodal planes at 8.1 MHz (upper) and 7.97 (lower) MHz. (B) Trapping of 6 µm fluorescent beads at 8.1 MHz. a to e: the growth of

We accomplished this with new transducers having a characteristic frequency around 7 MHz. With this PZT, ultrasonic standing waves are established around 8.1MHz and. notably, with three nodal planes. Shown in **Figure 4-6A** is the COMSOL simulation data showing the three nodes viewed from the side. The fluorescent images in **Figure 4-6B** are top-down views, and show the growing aggregate of trapped fluorescently-tagged sperm cells at the trapping site (**Fig. 4-6Ba-e**). Because this is a view from above, it is difficult to see the three nodes in the z-direction). **Figure 4-6Bf** shows the layered three planes with dotted line circles/ellipses outlining the trapping zones, which tended to be less irregular (more 'circular' in shape) than that observed with the 5 MHz transducer.

Measuring Resonator Channel Height

As mentioned earlier, the resonator cavity in this case is defined by the channel height, i.e., the space between the top and bottom glass layers (separated by laser-cut PDMS). The channel height (*h*) forms the resonator chamber whose dimensions are critical to the resonance frequency of the ultrasonic standing waves. The optimal resonance frequency, *f*, is proportional to 1/*h*, and with *h* defined by the thickness of the purchased film of PDMS, the optimal resonance frequency will change from chip-to-chip.

We have converged on a PZT whose characteristic frequency is ~8MHz, and this frequency requires a PDMS thickness of 280 µm. With this channel height and this PZT, 3 trapping nodes (on different 3D planes) are established in the acoustic standing wave. Our commercial source for PDMS could only provide a thickness consistency over several meters of film of 275 µm ±25 µm. This variance in thickness would require a optimal frequency that could range from 7.5-8.5 MHz, and use of a single frequency (e.g., 8.0 MHz) would, probabilistically, be ineffective for many of the chips. Consequently, it was essential to define the channel height for each fabricated chip so that a general frequency range can be established as a starting point for 'frequency sweeping'. This height measurement was accomplished using Fabry-Perot Interferometry, which essentially defines the chamber height through the refractive index of light delivered to the channel. As shown in **Figure 4-7A** , when the channel height was accurately defined, the optimal frequency could be extracted from the linear relationship between these two parameters. Having defined the optimal frequency for each chip, **Figure 4-7B** shows that frequency differences fall within a reasonably small range (±0.07 MHz), setting the stage for automated

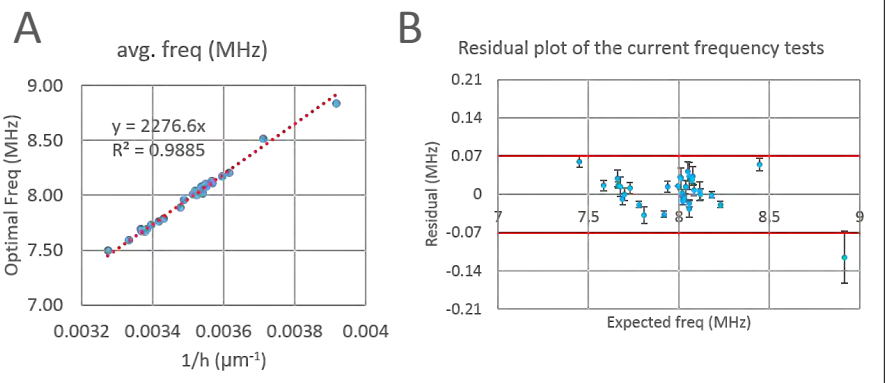


Figure 4-7. Linear correlation between optimal frequencies and channel heights (left)

frequency sweeping described in the next section.

Real-time Determination of Optimal Frequency

The first and, perhaps, most crucial step in the ADE process is a sweep through multiple acoustic frequencies for each chip. This accounts for any small chip-to-chip variation that may be present due to inconsistencies in material thickness or fabrication. The 'frequency sweep' applies a range of frequencies to the piezo, while 6 µm fluorescent beads are flowed through the trap site. After passing through the trapping site, the test bead solution is diverted to the waste chamber, reservoir 6. Custom software identifies the largest bead aggregation from 8 distinct trapping frequencies, and tags the optimal frequency based on aggregate size. That frequency is chosen for sample trapping throughout the rest of the run.

The actual optimal frequency is determined by running the fluorescent polystyrene beads through the resonator and then sweeping over a range of candidate frequencies using a starting point defined by the previously determined channel height (expected optimal frequency ± 0.07MHz) to establish successful trapping (**Fig. 4-8**). The optimal frequency is automatically selected by an algorithm that defines which frequency yielded the largest aggregation of beads. That optimal frequency f is plotted against $1/h$ to reveal the linear relationship where any data point outside of the acceptable interval (**Fig. 4-7**) indicates an outlier chip that is not used. To date, we have a 96% success rate in identifying the optimal trapping frequency within our predicted range based upon the previously observed relationship. It is imperative to identify the exact optimal trapping frequency so that the full complement of three nodes in the standing acoustic wave are formed. This maximizes the volume of sperm cells that can be trapped, while remaining within the ideal operational frequency for the piezo transducer.

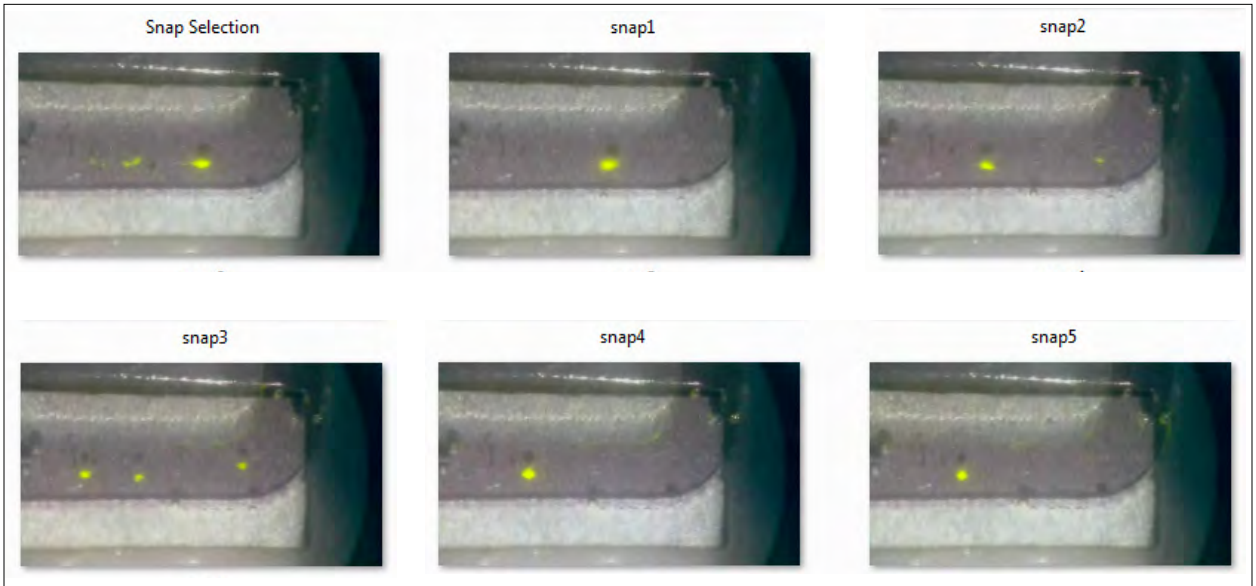


Figure 4-8. Video monitoring of the trapping process in real time. The progression from 'snap1' through to 'snap5' represents frequency sweeps from 7.95-8.05 MHz, with image capture after 5 sec of reaching the new input frequency. The extent to which beads have trapped (yellow) indicates the frequency optimum for that particular chip.

Low-rate Production of SONIC Chips in a Lab Environment

Lab-based low rate manufacturing is a temporary issue needed to develop, prototype, and optimize the SONIC system. Fortunately, all the components needed for chip production can be conveniently fabricated using laser ablation, which has a throughput that is adequate for the manufacture and quality testing of ~50-60 chips per week. Currently major components (PMMA reservoir layer, thin PDMS fluidic layer, PSA layer, glass cover layer) are generated in batches of 20 in each round of laser fabrication, followed by cleaning, alignment, and assembly. As shown in **Figure 4-9**, the total production time is estimated to be 2 hours and 45 mins per batch of 20 chips. This assembly time can be further reduced by automated large-scale laser cutting, as well as coordinated assembly by multiple researchers. Fabrication is straightforward to perform and can be scaled up for commercialization. Layer features are created via ablation of the channel outlines and via's using a CO_2 laser. The PDMS and glass surfaces are sterilized and activated via plasma oxidation before hand-assembly. The PMMA layer is capped with adhesive PDMS and then attached to lower glass layers with double sided adhesive. The PZT is bonded to the bottom of the chip with a small amount of ethyl cyanoacrlyate and allowed to dry prior to quality control testing of the chip. The final step of chip manufacturing is to treat reservoirs 4, 5, and 6 with a small amount (1 µL each) of Sigmacote, a siliconizing reagent that prevents backflow of sample into the chip after separation. This step is not time-sensitive, and can be performed in about 15 minutes at any point after chip assembly.

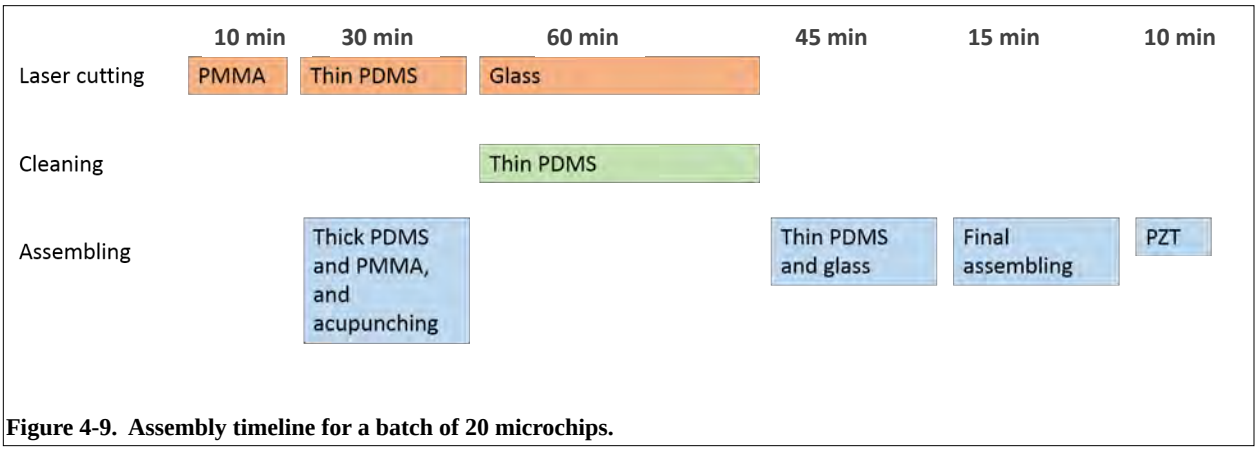


Figure 4-9. Assembly timeline for a batch of 20 microchips.

4B. Design, Fabrication and Testing of the Sonic Instrument

*The **SONIC** (**S**Ex **O**Ffender **N**Odal **I**Solation Of **C**Ells) System*

The Sonic Differential Extractor Mach-1 system (Fig. 4-10) is composed of several subsystems and components: (i) microfluidic cartridge, (ii) fluidic control, (iii) manifold interface for chip/transducer coupling, (iv) acoustic waveform/excitation source, and (v) optical imaging and analysis system. The entire system is currently controlled semi-autonomously via a laptop-driven custom software that was written in our lab. However, there is no fundamental reason why the system could not be configured to run fully-autonomously in the future. The following sections describe each of these subsystems in detail before discussing the final integration into an integrated, portable, field-use platform.

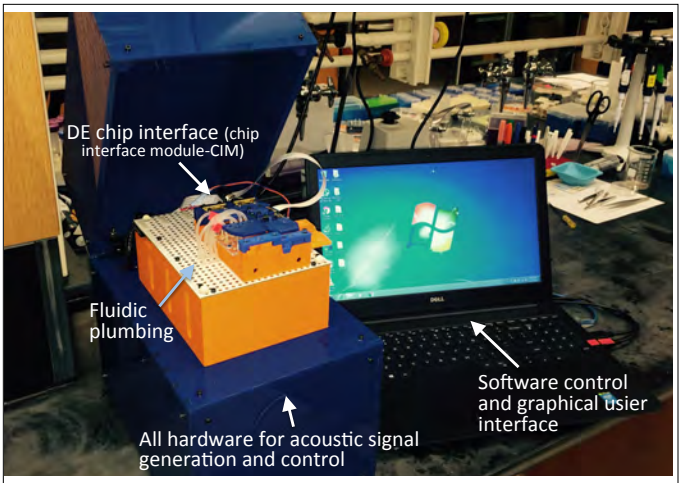


Figure 4-10: The final form factor for the SONIC differential

Microfluidic Cartridge Interface (CIM) and Fluidic Control

Compact syringe pumps pneumatically drive fluidic movement within the chip. Labsmith SPS01 syringe pumps are affordable (~$400) and have a small footprint for an off-the-shelf syringe pump, see **FIGURE 4-11.** Unlike the valving described by our lab [69,70 55,56] and other microfluidic researchers [76Grover; 77Quake 57,58], valving is done off-chip with Labsmith AV201 valves. The sequence of valve actuation was shown in **Figure 4-4**. The valving procedure, which is controlled by the software (described in a later section), involves a total of 5 steps. These are :

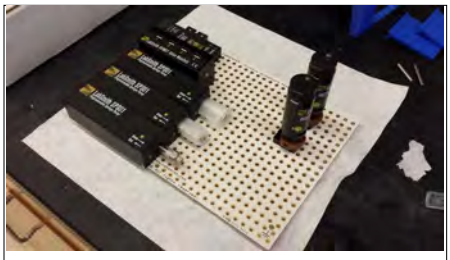


Figure 4-11. Fluidic pumping bread board. Bread board dimensions are 7.25”x5.25”. Shown are 3 SPS01 pumps with syringe tip visible. The two vertical objects are valves discussed in the next

(1) Add 110 µL bead solution, 65 µL sample solution and 43 µL wash/elution buffer to R1, R2 and R3, respectively. Place the chip underneath the manifold and close the manifold.

(2) Push 75 µL bead solution at 45 µL/min from R1 to R6, close the other path (This is for the test trapping for the verification of chip’s function)

(3) Push 50 µL sample at 45 µL/min from R2 to R5, close the other path (sample trapping)

(4) Push 13 µL wash/elution buffer at 45 µL/min from R3 to R5, close the other path (wash off the female lysate)

(5) Push 35 µL wash/elution buffer at 200 µL/min from R3 to R6, close the other path (sperm cell elution)

(6) Push 20 µL wash/elution buffer at 45 µL/min from R3 to R6, to clear the channel

Manifold Interface for Chip/Transducer Coupling

As with many of the parts used to assemble the SONIC prototype, the manifold is created through printing on a very cost-effective additive manufacturing process more commonly known as 3D printing (Ultimaker) using Polylactic Acid (PLA) as substrate, a biodegradable thermoplastic. This enabled rapid design iterations while saving on material/fabrication costs. A sideview of the chip interface manifold developed for the SONIC-Mach1 system is shown in **Figure 4-12**. The chip is loaded into the system by first resting it on the bottom manifold which is mainly a supporting flat

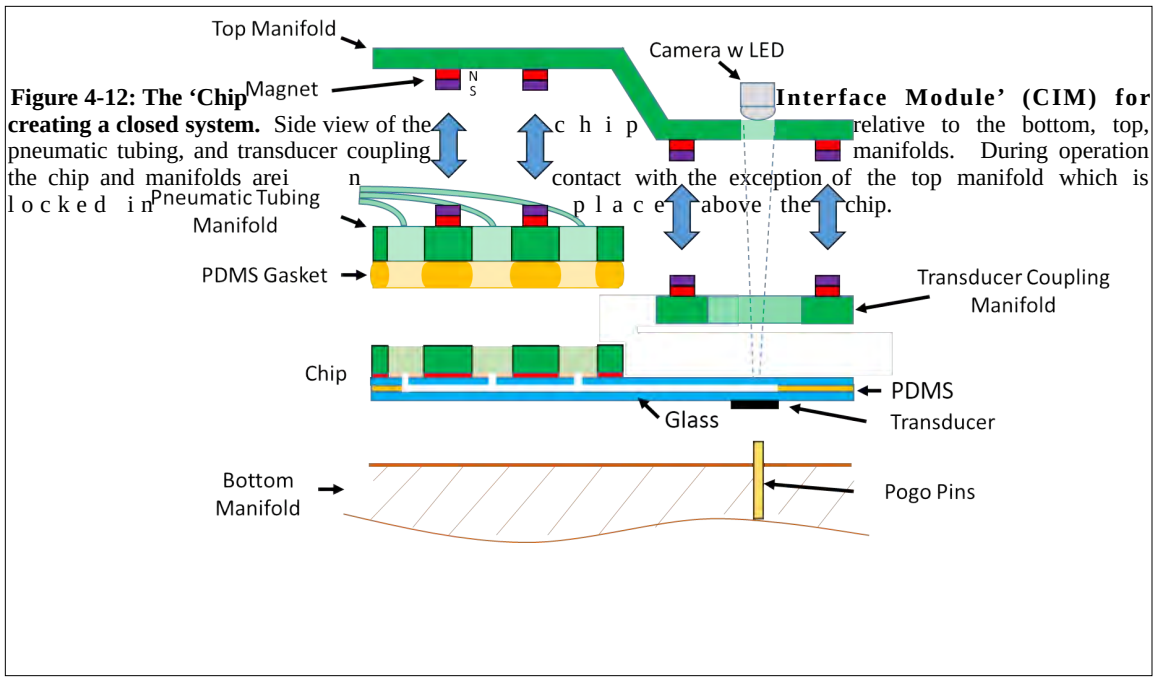


Figure 4-12: The 'Chip Interface Module' (CIM) for creating a closed system. Side view of the chip relative to the bottom, top, pneumatic tubing, and transducer coupling manifolds. During operation the chip and manifolds are in contact with the exception of the top manifold which is locked in place above the chip.

surface. This surface has one functional connection to the chip; it makes electrical contact to the PZT via protruding pogo pins, which are spring-loaded electrical contacts. This allows for the pneumatic manifold to be placed over the section of the chip containing fluidic reservoirs R1-R6. It is here that a PDMS gasket built in as part the manifold, allows for an airtight seal with the fluidic reservoirs. This part of the manifold also contains pneumatic tubing that is connected to the syringe pumps and valves. Finally, magnets are embedded into 3D printed recesses in the manifold to provide rigid clamping once the top manifold is locked in place. The transducer coupling manifold is placed on the section of the chip over the piezo to ensure good electrical contact between the pogo pins and piezo. The top manifold contains magnets used to clamp the previous components together as well as a CCD camera and LED light source to image and illuminate the trapping zone. The camera is used to capture video and still images of the trapping zone on the chip, enabling the user to observe bead and cell aggregation in real-time during trapping process. The LED matches the excitation wavelength of the fluorescent microfluidic beads, thus, enabling high contrast imaging of bead aggregation. Ultimately, a computer algorithm analyzes the images as the PZT sweeps

through eight different frequencies to determine the optimal frequency corresponding to the largest bead aggregation. This process is performed during each run prior to sample entering the fluidic architecture and transiting the trapping zone.

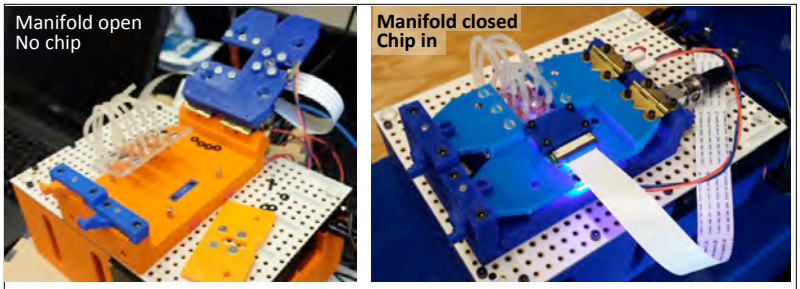


Figure 4-13. Open and closed SONIC system. The Manifold is open before

The primary benefit of using a 3D printed material is cost savings and part turn-around time, however, significant reduction in weight is also achieved. **Figure 4-13** shows the printed chip interface manifold; this is the area where the operator loads the chip for each run. Currently, it requires a few manual steps that, eventually, can be automated in future iterations of the instrument. At the present time, the operator loads the chip as shown in **Figure 4-14**. After loading, the clamping manifold is closed (hinged movement) and locked in place. When closed, an illuminating LED is automatically activated via a relay and the system is ready for operation using the GUI on the laptop. When the run is complete the operator is prompted to remove the chip, and the system is ready for a new chip, sample and analysis .

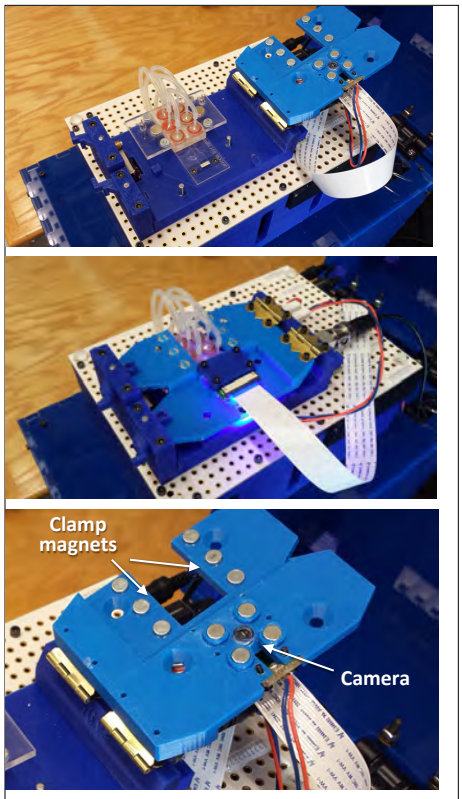


Figure 4-14. Loading a chip into the SONIC system. Image of a chip loaded into the CIM with the clamping manifold both open (upper image) and closed (middle image). The lower image shows the detail of the CIM with the magnets that provide passive clamping and the camera that

Acoustic Waveform/Excitation Source

The essential electronics in the SONIC system is a waveform generator, an amplifier and an oscilloscope; respectively, these generate the actuation voltage, amplify the signal and monitor the sperm cell trapping. For the waveform generator and oscilloscope, we excise the common, very bulky, bench-top hardware (Agilent's 33220A and Tektronix 1002B), which cannot easily be imbedded in an instrument, with Hantek's DDS-3X25 waveform generator and 6052BE oscilloscope. The advantages of the new electronics are: (1) more compact and portable, with roughly the dimensions of an ordinary textbook; and (2) interfacing with a PC through a USB connection and controllable by programmed user interfaces. The amplifier was home-built using a standard operational amplifier.

Smart Phone Camera-Monitoring of the Trapping Event

While trapping can occur without visual monitoring ('trap blind' mode), we include a miniaturized optical system for the monitoring of the trapping in progress. This is NOT needed to trigger action by the user, but provides a 'wellness check' for the ADE trapping system. Initially, we employed a smart phone coupled with the lens from an inexpensive laser

pointer to record progression of the trapping process, and the subsequent growth of the trapped aggregate of beads in real-time. **Figures 4-15** shows the hardware and **Figures 4-15B** the stills captured from a video during the trapping process with smart phone monitoring.

To further increase resolution as well as decrease instrument component costs, a stand-alone CCD camera was used to replace the smartphone **(Fig. 4-15C).** This camera has a number of advantageous characteristics. It has a high pixel count (5 Megapixels), allowing for better imaging of the bead/sperm cell aggregate. First, while not capable of imaging a single sperm cell trapped in the bead aggregate (as with a fluorescent microscope), the enhanced resolution is enabling. Second, and not insignificant, it has an extremely small form factor and low cost, thus, reducing instrument size and overall cost. Interface software was written to remotely connect the camera controller and initiate image capture – a key requirement for full software integration of the platform. After initial testing the as-shipped lensing system (**Fig. 4-15D**) was upgraded to further enhance resolution.

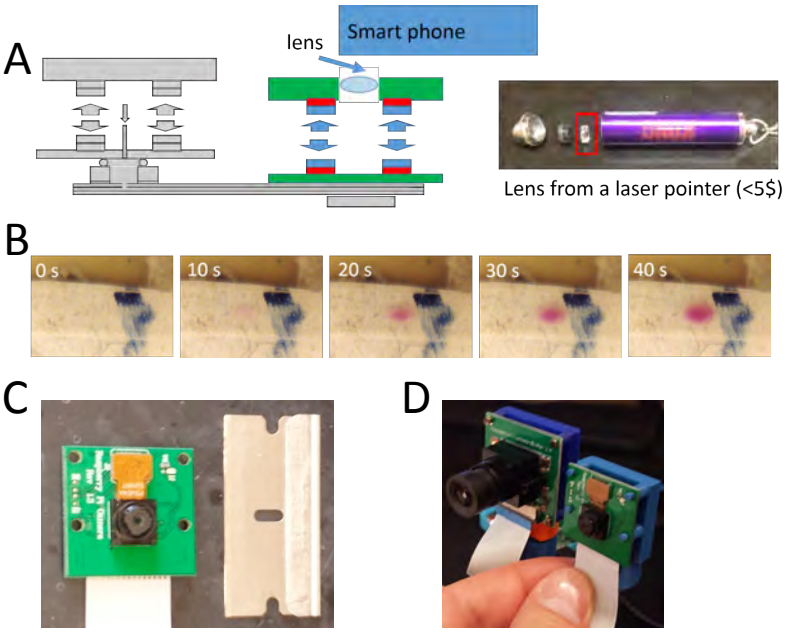


Figure 4-15. (A) A laser pointer lens and the digram of the smart phone-lens coupling through the manifold. (B) Real-time visualization of bead trapping. Video snapshots of 6µm violet beads trapped, recorded by a smart phone. Flow rate=30µL, frequency=8.4MHz, Vpp=21V. (C) **A stand-alone smart phone**

With this system, image comparisons can be made through use of a simple computer algorithm developed using a command line version of *ImageJ* called *Fiji*. Automated image analysis enables full automation of sample processing. **Figure 4-16** shows an original image, the processed image, which removes the background to highlight the aggregation, and finally the overlay of the original image and processed image, which verified that the algorithm was processing the image appropriately. The aggregation size is presented to the user as a number for comparison to other images providing a true metric for comparison. This number enables the

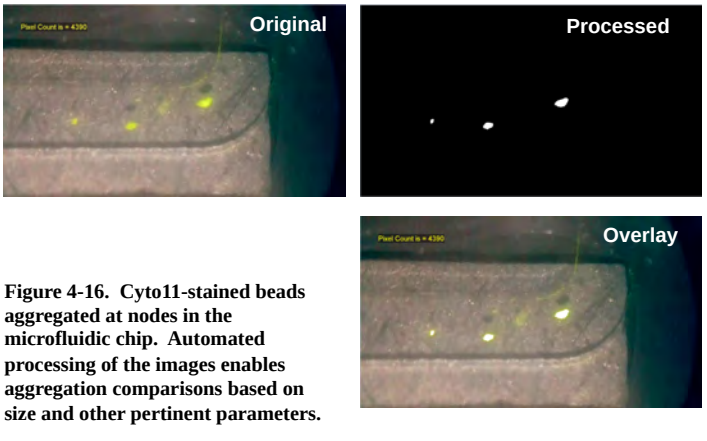


Figure 4-16. Cyto11-stained beads aggregated at nodes in the microfluidic chip. Automated processing of the images enables aggregation comparisons based on size and other pertinent parameters.

computer to make comparison calls autonomously, however, at present we still have a user make the final determination to rule out any anomalies that may occur as system development continues.

Development Of An All-In-One System

i) Design And Assembly

Figure 4-17. The SONIC instrument. (A) System Assembly with various components labeled. (B) The rear of the instrument has a normal AC power connector (120 or 240VAC) and laptop connections consist of 3 USB ports and 1

The subcomponents described in previous sections that were initially built into 'subcomponent' systems for proof-of-principle testing, were then assembled into a single portable platform. An 'exploded view' of the system is shown in **Figure 4-17-A** with various components labeled in the schematic that is coupled with a photo of the actual system. The operator only needs to interact with the cartridge interface module to load chips. This module is protected under a hinged lid for transportation; it is shown in its 'open' position in the figure. Unit AC power and communication cables to the accompanying laptop are connected via a 'patch panel on the rear of the instrument shown in **Figure 4-17-B**.

ii) Software and Control of the Trapping Process

All components, including the camera, are fully-integrated within a single GUI residing on a control laptop running LabVIEW software. The architecture already employed allows for easy

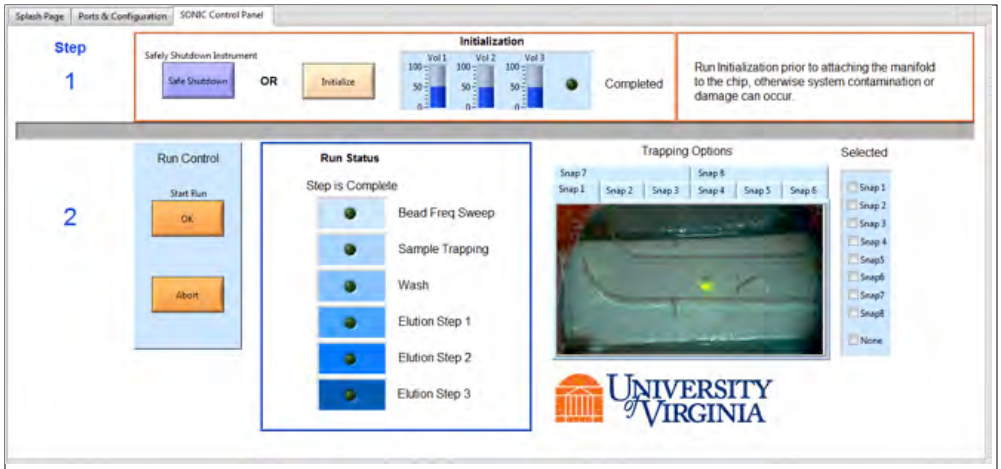


Figure 4-18a. A screenshot of the graphical user interface for the Sonic Differential Extractor

transition to fully autonomous operation from the current semi-autonomous state. The main control window of the GUI is shown in **Figure 4-18a.** This window is where the user controls the system/ process and observes the system operation in real-time. The GUI itself comprises a several button controls (*Initialize*, *Safe Shutdown*, *Start Run*, and *Abort*) and indicators let the user know which steps of the process have been completed.

The software is integrated with a usb connected barcode scanner to quickly load information stored with each microfluidic chip such as a chip serial number and optimal piezo driving frequency. At the conclusion of the run, a data folder is automatically created to track specific parameters used during operation, along with videos showing various stages of the run to aid post-run analysis.

Additionally, an Advanced User password has been implemented in the GUI for servicing needs. Once entered, additional control options are available to aid in troubleshooting system performance

Figure 4-18b. Screenshot of the GUI (graphical user interface) for the Sonic Differential Extractor video monitoring of the trapping process.

or for testing outside of the normal optimized parameters **(Fig 4-18b)**. This enables a dynamic interface that allows a technician to make a series of changes quickly and test them rapidly while keeping a normal user locked out from the advanced controls where they could unintentionally change parameters that would adversely affect performance. There are additional access codes that are only accessible to the developers - these are not needed by the user and allow access to a 'back panel' coding that is meaningful to a software engineer.

iii) Operation

Upon launching the software the operator is prompted to use a barcode reader to scan the number located on the microfluidic chip. In addition to manufacturing information the code on the chip contains the optimal frequency to operate the piezo at for cell trapping. The user is then taken to the main control window of the GUI shown in **Figure 4-18.** This window is where the user operates and observes the system operation. The operator first clicks the 'Initialize' button prior to loading

the chip in 'Step 1' or the operator can choose to shut the system down instead. System initialization ensures that the syringes are in the starting position. The operator then loads the chip into the system and selects the option to run in 'Step 2'. The system then begins running based on pre-programmed routines as well as values contained in a configuration file. This file contains parameters likely to change during development (syringe displacement, flow rates, etc.) enabling laboratory updates to be easily pushed into the field.

4C. TRAPPING WITH A LOW SPERM CELL NUMBER

Bead-Assisted Trapping

The physics of acoustic trapping has two quantitative limits - 1) in terms of the particle size, only particles >1 µm can be effectively trapped, and 2) there is a critical number of particles that must strike the trapping zone (node) over some finite period of time to order to initiate the formation of an aggregate. This latter limitation impacts the ability to trap sperm cells in cases where the total number of cells is low. It had been shown that 'seeding' the trap zones with beads prior to adding sample, allowed for the trapping of very low particle counts that could not be trapped in the absence of the beads [78 59]. This is illustrated in **Figure 4-19** where the efficiency of trapping drops precipitously with particle concentration. We leveraged previous work to create *'bead-assisted sperm cell trapping'* [79 60]. **Figure 4-20** shows this with trapping simultaneously monitored by both fluorescence and bright field microscopy. The bright points on the green background in the upper row, show cyto11-stained sperm cells - arrows in the far right image show single sperm cells as part of a 'darker' bead cluster. The beads, difficult to see in the fluorographs, are clearly visible in bright field mode (lower row) while the trapped sperm cells are not. Although studies are on-going, exploiting polystyrene beads can assist in the trapping of sperm from samples with concentrations

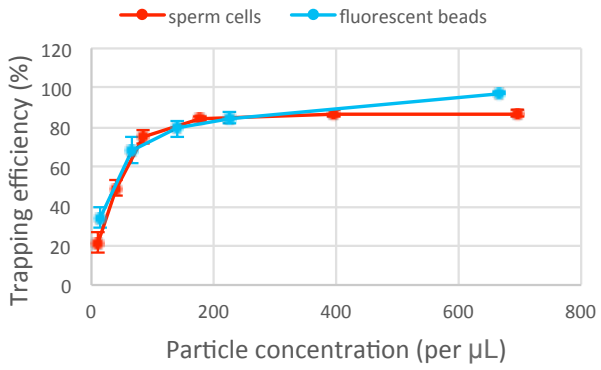


Figure 4-19. Dependence of trapping efficiency on particle concentration (n=3). Samples were infused at a flow rate of 30 uL/min and Vpp=12.

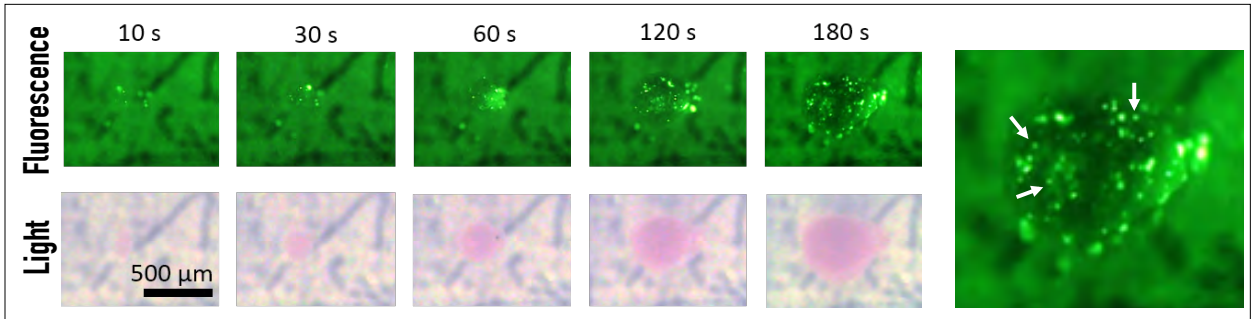


Figure 4-20. Visual monitoring of bead-assisted sperm trapping through both light and fluorescence microscopy. Still images from video of the trapping process shown at 10, 30, 60, 120, 180 seconds for both fluorescence (upper row) and bright

of <1 cell/µL (60 sperms cells in 60 µL), with the possibility of trapping a single sperm cell.

Effect of Trapped Beads on PCR

One of the concerns that we had early on was the possibility that release of the trapped sperm cells (into the male fraction reservoir) also releases the beads, and that this may be problematic with downstream PCR. We tested the effect of polystyrene beads on PCR in a number of formats including STR PCR and qPCR. The results for the qPCR studies are summarized in **Figure 4-21** where the effect of beads is minimal.

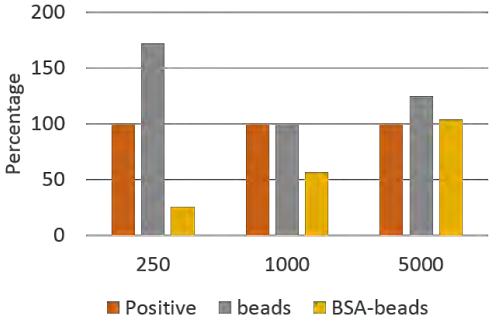


Figure 4-21. Visual monitoring of bead-assisted sperm trapping through both light and fluorescence microscopy.

4D. INITIAL CELL TRAPPING

Sperm Cell Trapping

Prior to testing mock sexual assault kit samples, it was demonstrated that sperm cells could be captured from a diluted semen sample. Neat semen from donor 5YOB (160,000 cells/µL) was diluted in water, with the addition of assisting beads. Samples containing 1,000, 500, and 100 total sperm cells were captured via SONIC, then extracted and amplified as described in the 'Methods' section. The lower trapping limit was determined to be 500 total sperm cells in a sample with at a concentration of 8 cells/µL.

Cheek Swab Mock Samples

To demonstrate operation under ideal conditions, mock samples containing 10,000 sperm cells, at a 1:1 ratio of female:male cells were separated via SONIC. Shown in the left panel of **Figure 4-22** is the pre-SONIC STR profile, which contains peaks representing both the female and male donors; the right panel is the post-SONIC profile, which is entirely male. Under these sample conditions, SONIC successfully

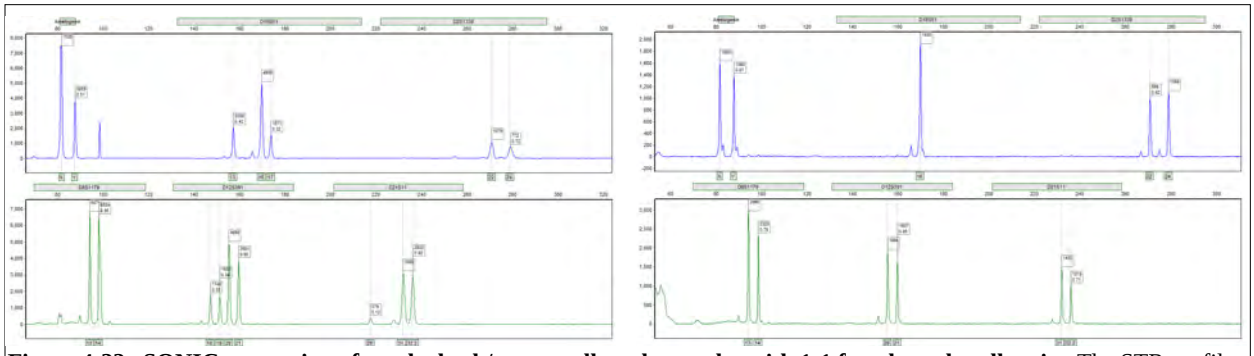


Figure 4-22. SONIC separation of mock cheek/sperm cell swab samples with 1:1 female:male cell ratio. The STR profile from a mixed sample (left) shows both female and male peaks. Peaks present from the female donor are X (amelogenin) 13, 17 (D18) 18, 24 (D25) 14 (D8) 18, 19 (D12) and 28, 32.2 (D21). Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The STR profile from the sperm fraction post-SONIC separation is a complete male profile, with strong peak heights and no female contribution.

isolated enough sperm cells to generate a complete male profile with peak height RFU (relative fluorescence units) over 1,000. Typical forensic data requires STR profiles to have peak heights > than 50 RFU, but this threshold varies from lab to lab.

Post-coital Vaginal Swab Mock Samples

The first batch of vaginal swabs provided from the UVA hospital came from 7 different donors, each of which had engaged in sexual intercourse during the previous 24 hours. In all cases there was no semen present on the swabs, all of which were cotton. Each donor provided two vaginal swabs, as well as two reference buccal swabs. After reconstituting the swabs in water and counting the number of cells (hemocytometer), diluted sperm cells were added to create samples that ranged in vaginal cells to sperm cell ratio from 3:1 up to 12:1. The pre-SONIC and post-SONIC STR profiles from every sample can be found in the **Figures S1-S8** (Appendix 2)**.** In every case the post-SONIC profile was entirely male, with all peaks matching the STR profile of the anonymous sperm donor. All samples tested in this study were liquid, and not dried overnight.

Dried Samples

In order to better replicate sexual assault kit samples, a known number of sperm cells were pipetted onto post-coital vaginal swabs and allowed to dry overnight. The female:male cel ratio could be approximated based on cell counts from the duplicate swabs provided by the donor. Successful

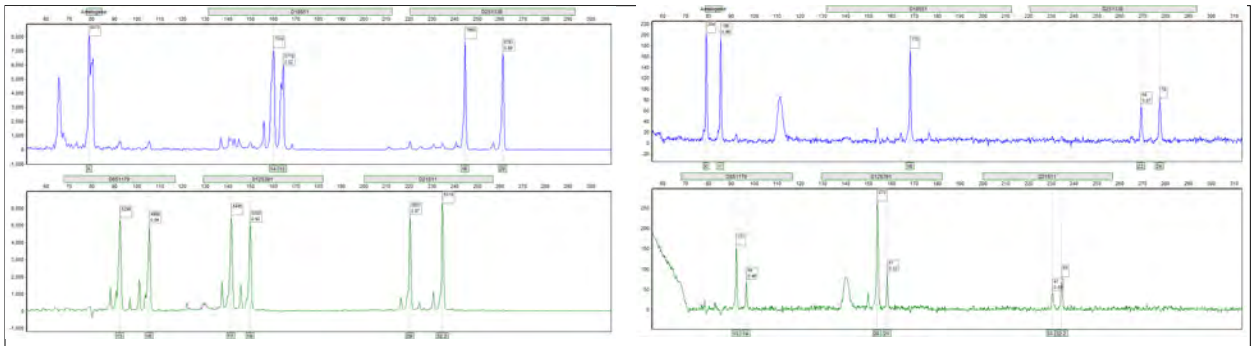


Figure 4-23. **Separation of sample with 15:1 ratio of vaginal cells:sperm cells.** The STR profile from a mixed sample (left) shows only female peaks. Peaks present from the female donor are X (amelogenin) 14, 15 (D18) 16, 20 (D25) 13, 16 (D8) 17, 19 (D12) and 29, 32.2 (D21). Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The STR profile from the sperm fraction shows all male peaks, but overall peak heights are very

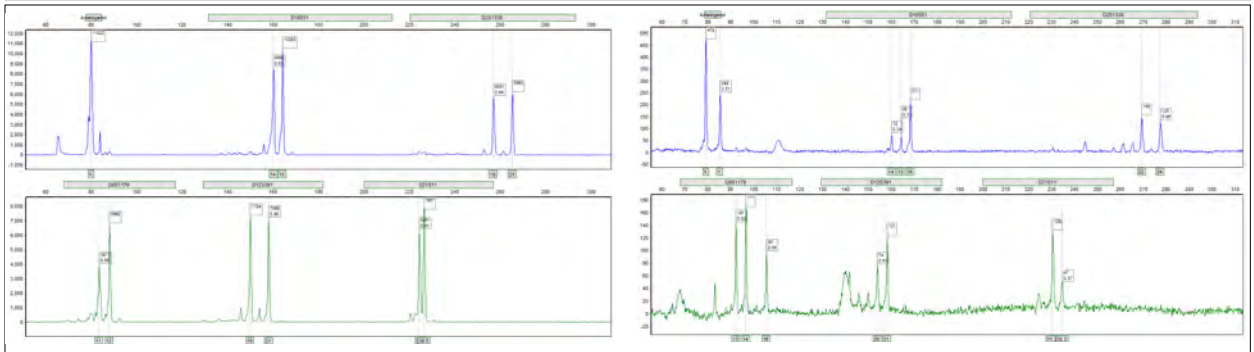


Figure 4-24. **Separation of sample with 20:1 ratio of vaginal cells to sperm cells.** The STR profile from a mixed sample (left) shows only female peaks. Peaks present from the female donor are X (amelogenin) 14, 15 (D18) 19, 21 (D25) 11, 12 (D8) 19, 21 (D12) and 30, 30.2 (D21). Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The STR profile from the sperm fraction shows two female peaks at D18, and overall peak heights

separation of sperm cells was achieved at ratios as high as 15:1 and 20:1 female:male cells (**Figs. 4-23 & 4-24**). The post-SONIC profile (right panels) for the 15:1 sample display male-specific peaks, while the 20:1 sample has two female peaks appear at 14 and 15 in D18. It is noteworthy that there was generic lab issue with PCR at this point (contamination at 110 in the blue dye and 140 in the green dye).

4E. Effect of Contaminants on Trapping

Common Sample Contaminants

There are multiple potential contaminants of concern with sexual assault kit swabs, including blood (either of the victim or perpetrator), yeast or bacterial cells. The most common way that yeast or bacteria would be present on the swab is due to bacterial vaginosis, defined as when the foreign cells are present at 20% the concentration of the host epithelial cells (source). To best replicate these conditions, activated yeast and live *Escherichia coli* cells were spiked into mock samples at 20% the concentration of donor epithelial cells.

Acquisition of Contaminants

A vaginal swab containing blood was obtained from an anonymous donor during their menstrual cycle. Using a hemocytometer and fluorescent dye, the cell count was determined to be 678 cells/µL, with heavy amounts of cellular debris present in the sample. Yeast cells were activated by adding 25 mg dry yeast to 500 µL water, and diluting 1,000X to reach a final concentration of 9,800 yeast cells/µL. Live E. coli cells were obtained from collaborators within the chemistry department, and diluted 100X to a final concentration of 2,600 cells/µL. Each substance was stored at 4C and used within one week of initial acquisition.

Samples Tainted with Blood

Samples contaminated with blood were formulated at a female:sperm cell ratio of 1:1 ratio, with an unknown initial amount of blood present on the swab. **Figure 4-25** shows the STR profile prior to SONIC separation (left panel), and the resultant sperm fraction profile post-SONIC (right panel). The

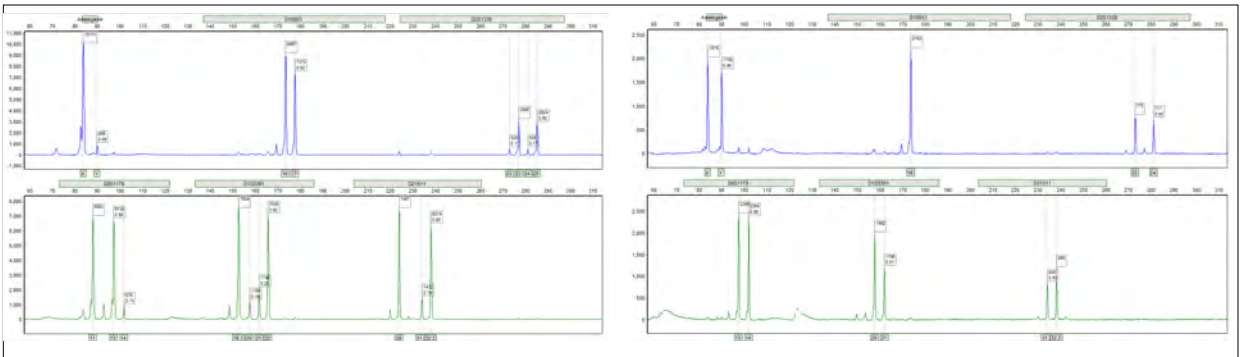


Figure 4-25. **Separation of 1:1 sample contaminated with blood.** The STR profile from a mixed sample (left) shows both male and female peaks. Peaks present from the female donor are X (amelogenin) 16, 17 (D18) 23, 25 (D25) 11, 13 (D8) 18.3, 21 (D12) and 29, 32.2 (D21). Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The STR profile from the sperm fraction shows a complete male profile with strong peak heights.

post-SONIC profile compares favorably to that obtained with the isolated sperm fraction from a 1:1 sample without blood, indicating that the presence of blood in a sample did not inhibit trapping.

Samples Tainted with Yeast

Samples containing activated yeast were formulated with 5,000 epithelial cells, 5,000 sperm cells, and yeast cells ranging from 1,000 - 3,000 (20-60% epithelial cell concentration). The results from

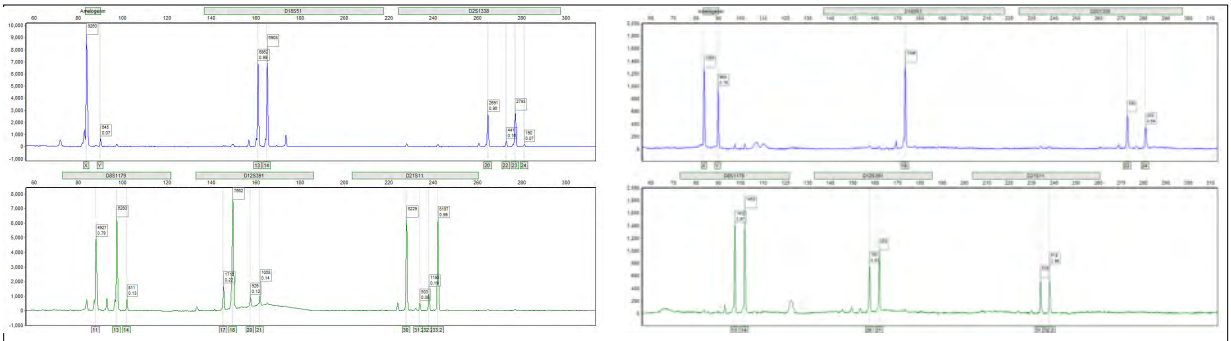


Figure 4-26. **Separation of 1:1 sample contaminated with yeast.** The STR profile from a mixed sample (left) shows both male and female peaks. Peaks present from the female donor are X (amelogenin) 13, 14 (D18) 20, 23 (D25) 11, 13 (D8) 17, 18 (D12) and 30, 33.2 (D21). Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The STR profile from the sperm fraction shows a complete male profile with strong peak heights.

the 40% sample are shown in **Figure 4-26** (left panel), with a full male profile obtained from the sperm fraction after trapping via SONIC (right panel). Hence, there appears to be no apparent inhibition of sperm cell trapping in the presence of yeast at biologically-relevant concentrations.

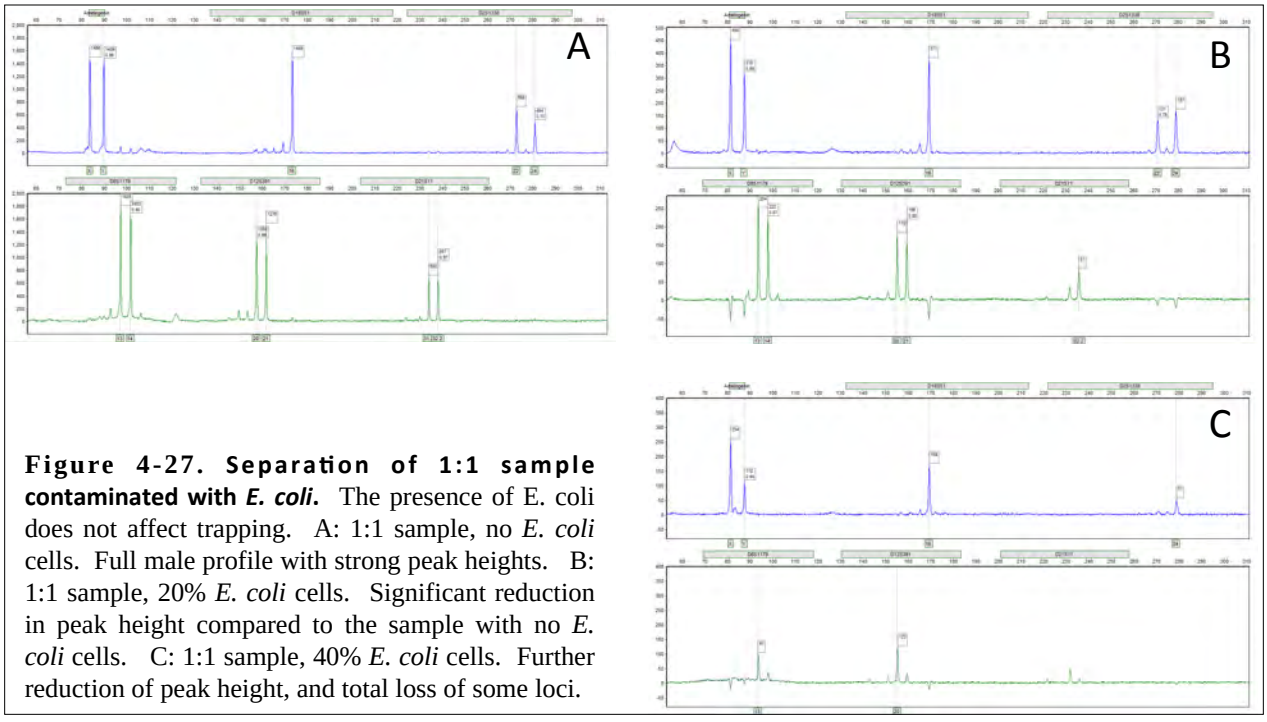


Figure 4-27. Separation of 1:1 sample contaminated with *E. coli*. The presence of E. coli does not affect trapping. A: 1:1 sample, no *E. coli* cells. Full male profile with strong peak heights. B: 1:1 sample, 20% *E. coli* cells. Significant reduction in peak height compared to the sample with no *E. coli* cells. C: 1:1 sample, 40% *E. coli* cells. Further reduction of peak height, and total loss of some loci.

Samples Tainted with E. Coli

Samples that were spiked with live *E. coli* cells were also formulated at 5,000 epithelial cells, 5,000 sperm cells, and yeast cells at 1,000 or 2,000. The results from samples with no *E. coli (A)*, 20% *E. coli* (B), and 40% *E. coli* cells (C) are shown in **Figure 4-27**. It is apparent that, as the concentration of *E. coli* increases, the capture efficiency of sperm cells decreases. We hypothesize that this is due to the size of *E. coli* cells (2-3µm in length), is comparable to sperm cell size (5-6 µm in diameter) and, thus, there may be competition between *E. coli* and sperm cells for trap site occupancy.

4F. TRAPPING UNDER OPTIMIZED CONDITIONS

Sperm Viability Testing

We observed that STR profiles from the isolated sperm fraction from SONIC-treated samples consistently had abnormally low peak height, particularly given that we knew the number of sperm cells added to the sample. By determining the capture efficiency of sperm cells in each reservoir through qPCR measurements, we determined that less than 35% of the sperm cells added to the initial sample were accounted for in the final concentration of male DNA. This prompted a closer look at the sample make-up, leading to a comparison of the neat semen aliquots. **Figure S-8** (Appendix 2) shows the STR profiles from 1,000 sperm cells from the first and second aliquots of neat semen from donor 5YOB. From this, we concluded that the initial semen sample had degraded significantly over time, and as a result, the number of sperm cells added to each sample was largely overestimated.

Reduction of DNase Concentration

Concern about additional contribution to low RFU profiles was the mass of DNase added for post-SONIC processing of the male fraction. It is possible that excess DNase inhibit amplification of male DNA, especially if not heat-killed effectively, or if some sperm cells are prematurely lysed. The DNase I/DNase buffer concentrations were reduced to 25% of their original concentration, which didn't negatively impact removal of female DNA, but showed improvement in male peak height.

Removal of D18 primer

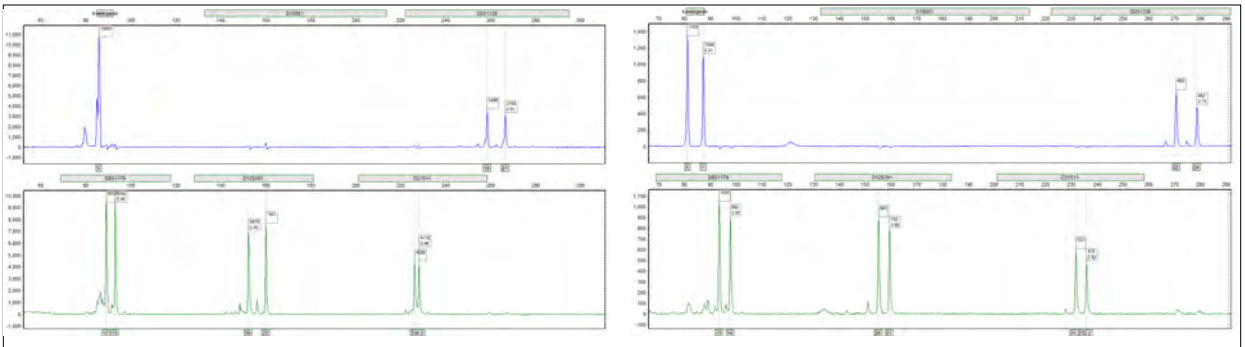


Figure 4-28. 20:1 sample trapped with updated protocol. (Left) The STR profile from a mixed sample shows only female peaks. Peaks present from the female donor are X (amelogenin) 19, 21 (D25) 12, 13 (D8) 20, 22 (D12) and 30, 30.2 (D21). **(Right)** The profile from the sperm fraction shows only male peaks, with high RFU. Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The male profile from a 15:1 sample

Our collaborators in primer design for the lower-plex STR kit at Promega suggested that the D18 primer be removed from the custom 6-plex primer set that had been used to this point. Their observations were that absence of the D18 primers led improved peak height, as well as reduced inhibition. To test this, the exact same sample was amplified using the original 6-plex primer set (Amel, D18, D25, D8, D12, and D21) and the 5-plex primer set (Amel, D25, D8, D12, and D21). The resulting profiles (**Fig. S-9;** Appendix 2) showed significant improvement in peak height at all loci when amplifyed with the 5-plex primer kit. With these changes implemented into the new protocol (fresh sperm sample, lower DNase I concentration, 5-plex primer set), a 20:1 sample was separated via SONIC and amplified, with the pre-SONIC (left panel) and post-SONIC (right panel) profiles shown in **Figure 4-28**. The pre-SONIC profile is dominated by female DNA, while the post-SONIC profile is fully male, with excellent peak heights at all loci.

Recovery of Failed Trapping

Occasionally, we would observe poor trapping as a result from either software or hardware malfunctions. When this occurred, either the sperm cells were not separated from the female fraction at all, or they trapped (aggregate formed in trap site) but prematurely lost from the trap zone. In both cases, the sperm cells ultimately flowed to reservoir 5 (instead of R4), the non-sperm cell reservoir. While not a viable long-term solution, the fluid in R5 could be recovered from the chip, spiked with additional 'assisting' beads, and then re-trapped on a new chip, at which point, we observed full recovery of the sperm cell fraction. This has been demonstrated successfully with samples as high as 30:1 that failed the initial trapping process (**Fig. S-10;** Appendix 2).

4G. TRAPPING SPERM CELLS IN PRESENCE OF HIGH CONCENTRATION OF E-CELLS

Consistent with information obtained in discussion with experts in multiple state and federal labs, sexual assault samples are diverse in nature, tend to be unpredictable in terms of abundance of sperm cells and epithelial cells, or unknown cellular contaminants. Given the diverse opinion, we chose to analyze mock samples (both liquid and swab) ranging in **female**:**male** cell ratios and including **1:10, 1:1, 5:1, 10:1, 20:1, 40:1** and **100:1**.

40:1 Female:Male Cell Ratios

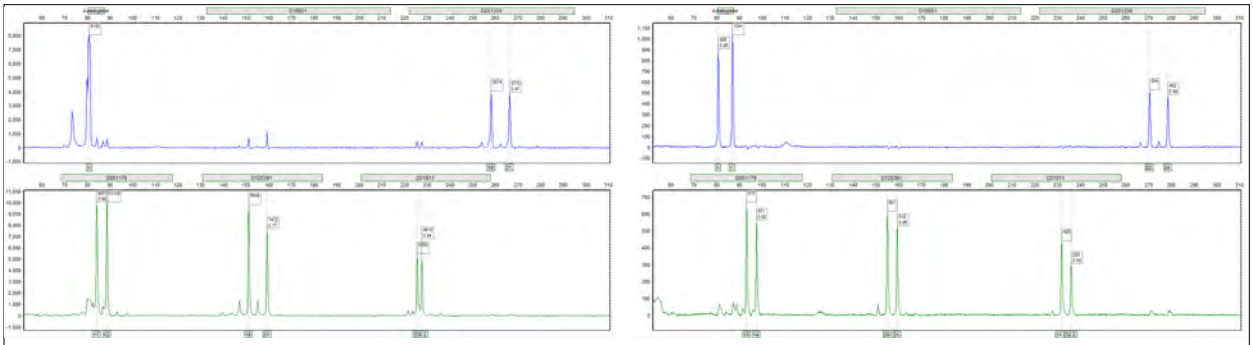


Figure 4-29. **Separation of sample with 40:1 ratio of vaginal cells:sperm cells.** The STR profile from a mixed sample (left) shows only female peaks. Peaks present from the female donor are X (amelogenin) 19, 21 (D25) 11, 12 (D8) 19, 21 (D12) and 30, 30.2 (D21). The profile from the sperm fraction (right) shows only male peaks. Peaks from the male donor are X, Y

With the aforementioned changes in protocol showing a positive influence, samples were prepared that would test the higher ranges of female:male ratios. Using the newest post-coital vaginal swabs obtained from the UVA medical center, two 40:1 samples were prepared that contained 500 and 1,000 total sperm cells, respectively. The pre-SONIC and post-SONIC profiles are shown below for the 500 sperm cell sample (**Fig. 4-29**); showing a complete male profile from the amplified sperm fraction.

100:1 Female:Male Cell Ratios

Having achieved the highest ratio separation to date, 100:1 samples were prepared to test the limits

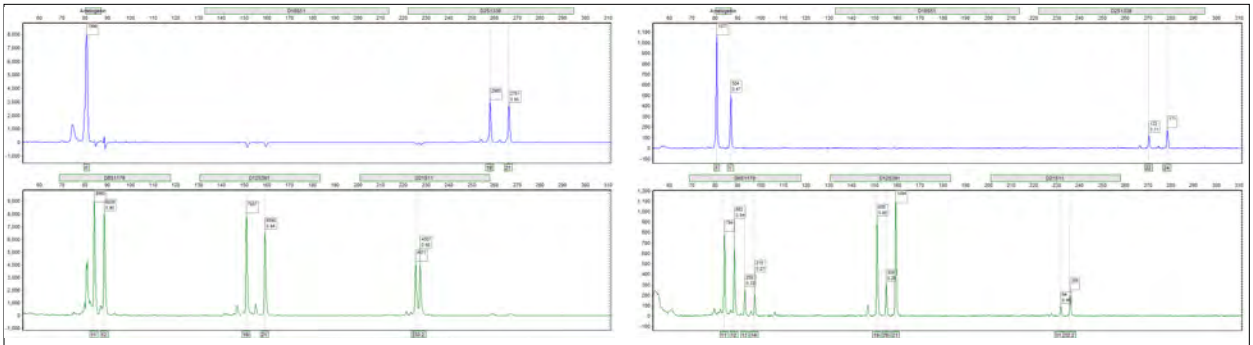


Figure 4-30. **Separation of sample with 100:1 ratio of vaginal cells:sperm cells.**The STR profile from a mixed sample (left) shows only female peaks. Peaks present from the female donor are X (amelogenin) 19, 21 (D25) 11, 12 (D8) 19, 21 (D12) and 30, 30.2 (D21). The profile from the sperm fraction (right) shows only male peaks. Peaks from the male donor are X, Y (amelogenin) 16 (D18) 22, 24 (D25) 13, 14 (D8) 20, 21 (D12) and 31.2, 32.2 (D21). The STR profile from the sperm fraction

of the SONIC instrument. These samples contained 25,000 female epithelial cells and 250 total sperm cells. In **Figure 4-30** the pre-SONIC and post-SONIC profiles are shown for a 100:1 sample in which a full male profile is obtained with strong peak heights, and minor female contribution at the D8 locus. While not an isolated male profile, this result is highly promising and shows that separation of sperm cells from a 100:1 sample may be feasible with the SONIC instrument.

20:1 Female:Male Cell Ratios Using PP18D

Since the 5-plex STR chemistry used here is not a viable (or available) STR kit for forensic labs, it was imperative that the SONIC-separated fractions from a mock sample be analyzed (PCR/ electrophoresis) with a more accepted STR chemistry. We evaluated this with Promega's PowerPlex 18D STR kit. This challenges the SONIC DE in terms of assuring that:

1) the pre- and post-SONIC treatment does not lead to inhibition in the PCR step

2) there was no preferential effect on any particular locus

3) an acceptable peak balance is maintained

4) there was no preferential effect on longer vs shorter STR fragments

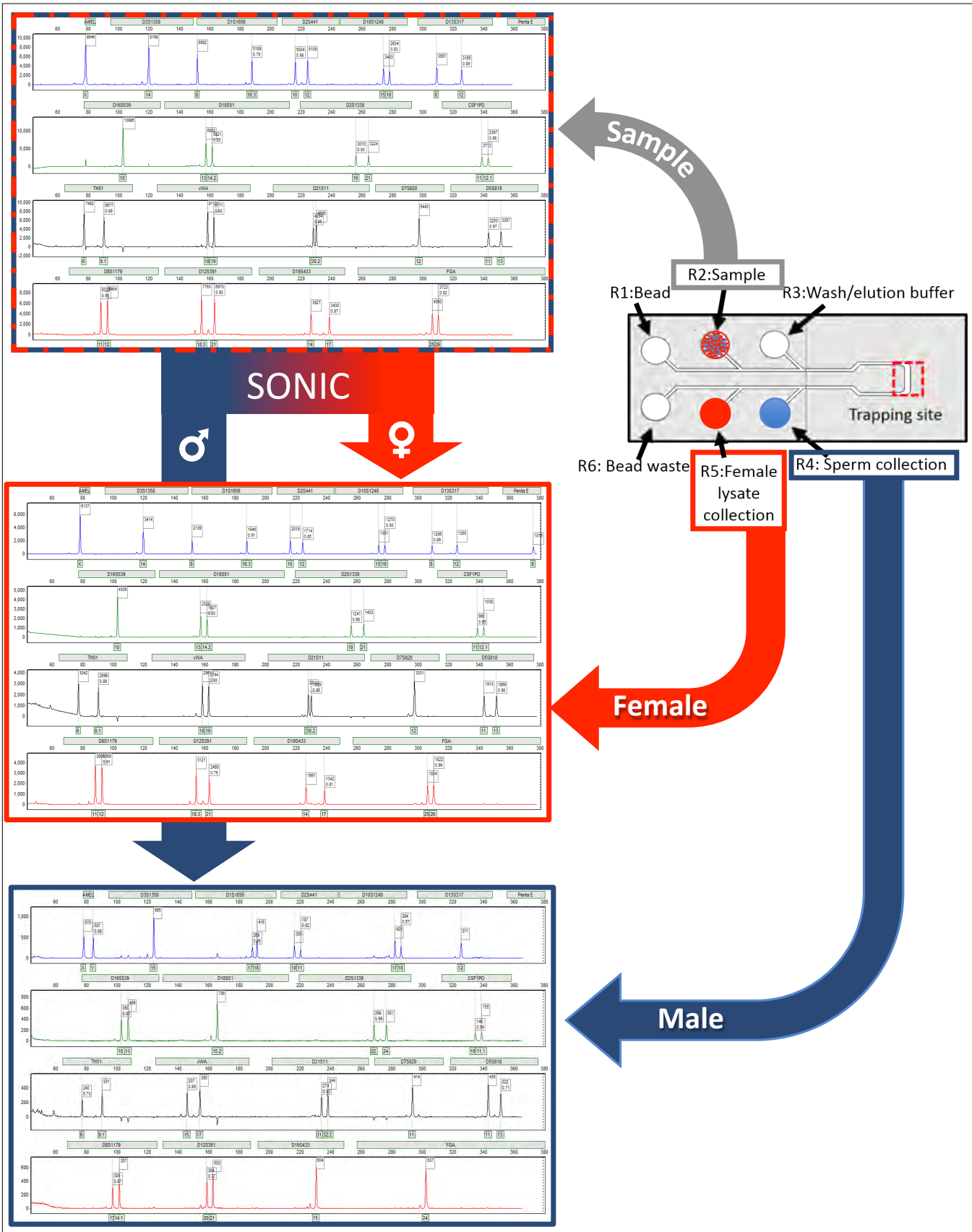


Figure 4-31. SONIC processing of a 20:1 (F/M cell) swab sample. PCR and electrophoretic analysis of the male and female

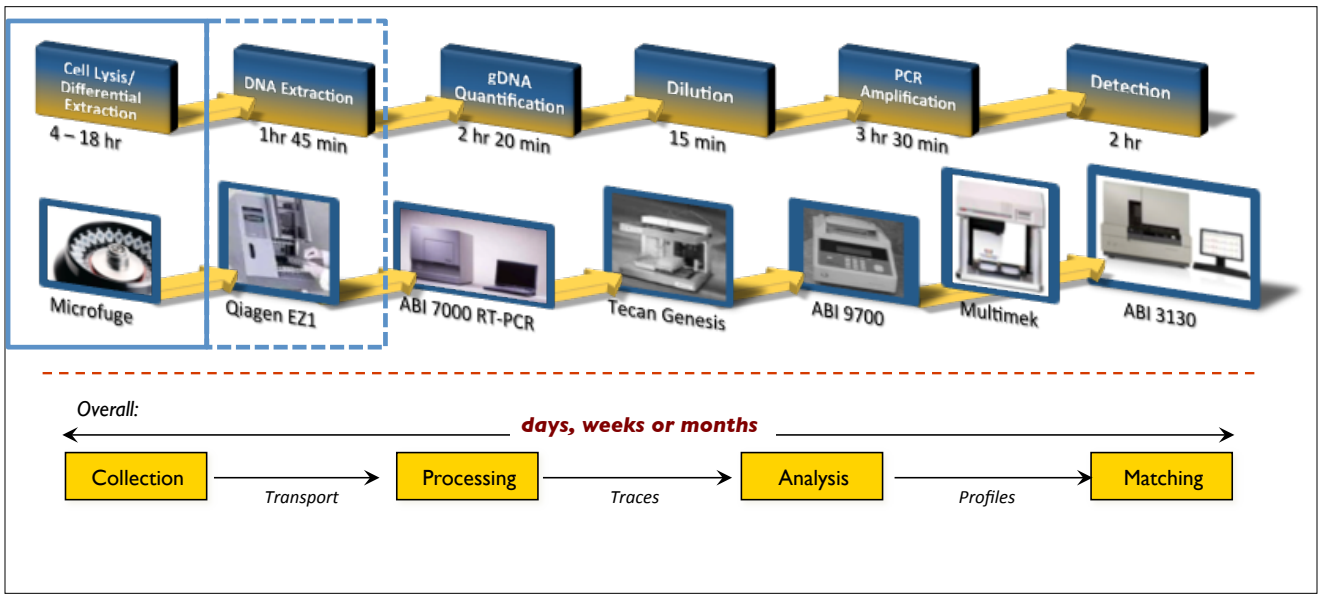
This experiment used a 20:1 female:male sample composed of post-coital vaginal cells spiked with sperm cells, and was separated with the most recent protocol. Total experimental time, including sample prep, instrument operation, and post-SONIC chemistry was 60 minutes to achieving PCR-ready male DNA.

The results in **Figure 4-31** were extremely encouraging, as a pure male profile with strong peak heights at all loci was obtained from the male fraction of the chip. This can be directly compared to both the pre-SONIC mixed profile, and is completely distinct from the female profile obtained from the non-sperm reservoir of the SONIC chip. This confirms that our instrument and separation protocol will conform to the back-end chemistry currently utilized in forensic labs around the country. The positive control for the male donor is given in **Figure S11**.

5. DISCUSSION AND CONCLUDING REMARKS

5A. ACOUSTIC DIFFERENTIAL EXTRACTION

We live in an era where the umber of DNA samples that will be processed annually continues to escalate and, presumably for the foreseeable future, will continue to do so. With this back drop, it is clear than any methodology that can help expedite any part of the 5-step DNA analysis process (Fig. 5-28) is worthy of consideration. Development of a SONIC (**S**EX **O**FFENDER **N**ODAL **I**SOLATION OF **C**ELLS) System was proposed as an unprecedented analytical system for cell sorting, with the demonstrated capability of trapping sperm cells in the presence of a sea epithelial cells. This project, obviously, focused on sexual assault samples (mixed female cell/sperm cell samples) and, in particular, the first step of the process illustrated in **Figure 5-1**. In fact, the first two steps are impacted by the SONIC methodology, as extraction of DNA from the male fraction has been included in the overall method, thus, yielding a process that allows for DNA sample processing from swab-to-PCR-ready DNA in less than 1 hr.



The project effort was primarily focused on the building of SONIC prototypes for the trialing of the Acoustic Differential Extraction (ADE) methodology in forensic laboratories. Three candidate labs were identified early-on and had agreed to run 20-30 *bona fide* samples. However, the effort overstretched into a 1 year no-cost-extension period, primarily because of delays in hiring a talented mechanical engineer to assist in the 3D design (SolidWorks), 3D printing hard mount parts, sourcing of electronic components, assembly of those parts into a functional instrument and, finally, testing of the instruments.

5B. *SONIC DE-MACH1* SYSTEM

THE SONIC MICROFLUIDIC CHIP

While not discussed in this report, flow rate impacts efficiency of the SONIC system substantially. In the late 1950's, van Deemter showed that flow rate in HPLC played a significant role in the efficiency of the separation (resolution, number of theoretical plates, etc.) [60]. They attributed this to the time needed for an equilibrium to be established between analyte in the mobile and stationary phase. Acoustic trapping is not unlike chromatography in this respect, primarily because the strength of the trapping force (F_r; axial radiation force) determines how effective particle trapping will be in a flow stream. If high flow rate is coupled with low F_r, trapping can be very inefficient. A more macroscale analogy might be the difficulty in attempting to extract someone from a flowing river – the faster the flow the more difficult it is to capture them. The trapping force is affected by a number of parameters (described in the *Introduction* section), and under certain conditions, we were able to trap at flow rates as high a 100 µL/min, allowing for mL-scale samples to be loaded into the system in tens of minutes. That notwithstanding, we settled on an optimal flow rate of 30 µL/min, which allowed for the sample (swab cell lysate) volumes used here to be loaded in two minutes.

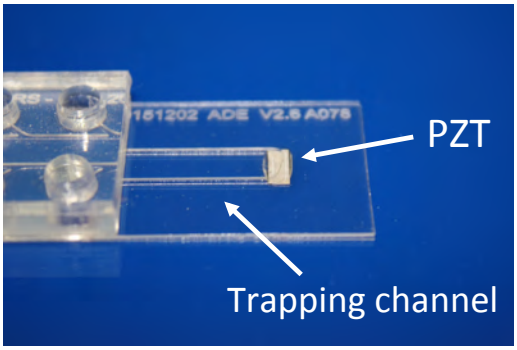


Figure 5-2. The SONIC chip.

The current SONIC chip (**Fig. 5-2**) design requires that 'sample' that is loaded into the R2 reservoir have intact sperm cells in a lysed epithelial cell environment. This is currently achieved off-chip with a custom chemistry protocol defined for this system, however, it is reasonable to anticipate that this process could be incorporated into a future design, in concert with some minor hardware modifications. Similarly, the male fraction that results in R4 post-SONIC treatment has intact sperm cells with minimal (if any) female DNA. This fraction could be funneled directly into any given DNA extraction process a lab chooses to use. However, we have developed a post-SONIC chemistry that 'extracts' the male DNA into PCR-ready form in ~10 min. This is available to the user, should they choose to use.

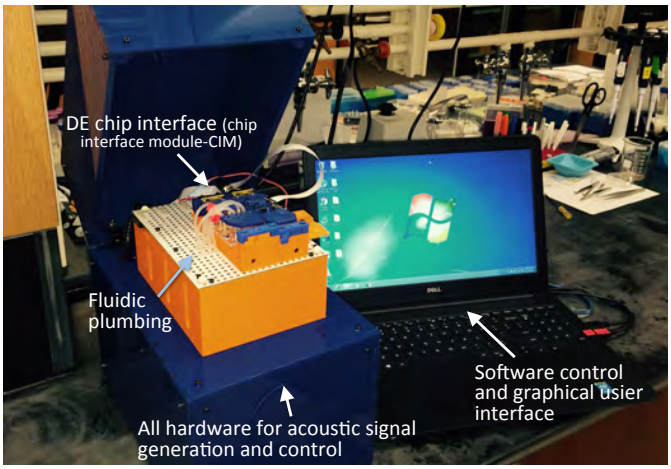
In terms of the chip, the sourcing of miniaturized PZTs that were ~2 mm (w) x 4 mm (l) x 1mm (d) in size was advantageous on several fronts. First, their inherently small size (reduced by order of magnitude over the PZTs we utilized in earlier work [9]), allowed it to be shifted from being part of the instrument and, instead, bonded to the chip. Related to this is the second advantage, that is, with a PZT cost of ~ $1.50 (USD), it can be included as part of the chip during fabrication, and hence, a component of the single-use, disposable consumable.

A current functional limitation of the chip is the capability for analysis of only a single sample. This was purposeful and exactly what we proposed in 2013 - prove out i) the concept of separation, ii)

the hardware and 3) the user executability for a single sample .... before designing a multiplexed chip. Our discussion with forensic labs and analysts on this point yielded some interesting feedback. Some saw this as an Achilles heel, indicating that they likely would need to run more than a single sample, while others indicated that they see significant potential for expediting single sample analysis under 'rush' conditions.

THE SONIC INSTRUMENT

The current instrument (**Fig. 5-3**) has a number of capabilities that we had not built into previous prototypes. A stand-alone cell phone camera that allows the user to visualize the trapping process in real time is valuable. While this does not require the user to 'attend' the instrument during the trapping process, a cursory glance allows the user to know that trapping is functional, with or without the presence of sperm cells in the sample. Again, with the goal of cost-effectiveness, the miniaturized camera components can be purchased off-the-shelf at roughly $35 (USD).



The software is currently laptop-driven and composed of two parts - a graphical user interface (GUI) for the user, and back-panel code that runs the system from sample input to male fraction collection. The GUI is designed to be user-friendly and allows the analyst in input critical sample information, monitor icon indicators that show the progress of the trapping process, as well as a screen connected to the embedded camera realtime trapping observation. The back-panel code in not accessible to the user (but is to the developers) and, essentially, controls all other functions. It executes the code that controls the entire automated fluid flow/trapping process, while also doing specific 'wellness' checks to assure all components are functional. If we were to design and build a *Mach-2 version* of the instrument, we would supplant the laptop with by a micro-controller and an embedded LCD screen with touch control, which would actually be lower in cost than a laptop.

Interestingly, the current BOM (bill of materials) for the instrument is <$7K - more than half of this cost (~$4K) is associated with the pneumatic fluid control system that is purchased from LabSmith Inc. It is reasonable to expect that sourcing more cost-effective parts from Asia could drop this cost to $1-2K; yielding an instrument BOM of <$5K, and presenting the possibility that a commercial product for forensic labs could be in the $10-15K range. This would give the SONIC system a stealth advantage of potentially being very affordable.

THE SONIC PROCESS

In order for the SONIC process to be effective, we had to create both pre- and post-SONIC processing chemistry. A specific pre-SONIC chemistry essential to the process prepares the sample for loading into the chip in a form that optimizes sperm cell isolation and assures that the only particulate components in the sample be sperm cells. As a result, samples (containing cells eluted from a swab) are treated in a manner comparable to standard differential extraction – that is, the non-sperm components are lysed. This process involves a ~10 min pre-SONIC.

In terms of the post-SONIC chemistry, the male fraction that consists of intact sperm cells and buffer, presumably, with little or no female DNA. The illustration in **Figure 5-4** shows both the steps and the instrumentation involved in SONIC processing of a sample. The user can choose processing as:

i) 'Swab-to-male fraction' ready for extraction - if this is preferable, the SONIC can be incorporated directly into the DNA extraction method of choice that is part of the standard workflow for the lab.

or

ii) 'Swab-to-male DNA' ready for PCR - this is an alternative process and NOT an absolute requirement for SONIC-based ADE. Here, a single step temperature-dependent treatment yields PCR-ready male DNA for analysis. There is chemistry (again, ~10 min) that processes the isolated male fraction so that it contains the male DNA in a form that is ready for direct-to-PCR analysis.

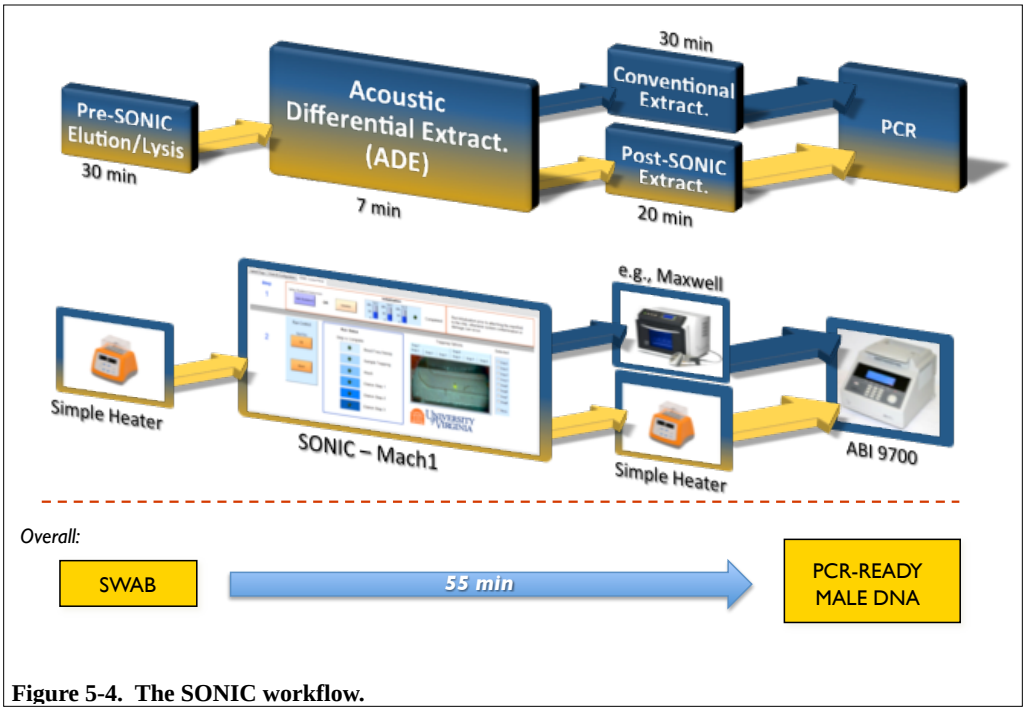


Figure 5-4. The SONIC workflow.

In summary, we have been successful in defining the SONIC system - a microfluidic chip, the corresponding instrument and the necessary chemistry - that, in combination, is capable of processing a sperm/female cell mixture from swab-to-PCR-ready male DNA in less than 1 hour. While this indicates that the goals of this project have been met, only through the trialing of this technology in credible forensic labs will we know whether it has merit, and is worthy of consideration of a phase 4 which would involve productization. Evaluation of the effectiveness of this SONIC system was carried out in two forensic labs - Dr. Kim Meza at the Mesa Police Forensic Services (Arizona) and Dr. Cecelia Crouse at the Palm Beach County Sherriff's Office Crime Laboratory. The details and results of these evaluations are presented in section 6.

6. External Evaluation of the In-Lab Testing of the SONIC System

THE MOTIVATION

The development of the differential extraction system at the University of Virginia has been efficient and successful, but a true external evaluation was necessary to determine the effectiveness of this technology with 'real' samples. At UVA, we were limited to testing only mock sexual assault samples, although we gathered as much information as possible to generate the most realistic mock samples prior to external testing. As a result, collaborations were set up in advance with the Palm Beach County Sheriff's Office (Palm Beach, FL) and the Mesa Police Department (Mesa, AZ), where we were able to place a prototype instrument in each lab, spend 1-2 days training select analysts in the specific operation of the instrument, and then observed them testing real casework sexual assault samples. The instruments were left for 1-2 months for use by our collaborators when they had time and samples to test.

PREPARATION FOR EXTERNAL TESTING

Prior to instrument placement, 20 fresh microfluidic chips were fabricated for each lab. Each chip underwent quality control testing to ensure adequate trapping performance, and was packaged in vibration-dampening foam for shipping. Fresh reagents for cell lysis, DNA extraction, and PCR amplification were aliquoted and shipped at -20 C to each lab prior to our arrival. The instruments themselves were packaged and carried-on flights for transport.

TESTING AT THE PALM BEACH COUNTY SHERIFF'S OFFICE

Testing at PBSO in Dr. Cecelia Crouse's lab was organized and overseen by Ms. Julie Sikorsky and carried out by Celynda Edwards and Brandy Plean. The dates of testing were January 30th-February 1st, and allowed for extensive training of the analysts as well as sample selection for testing. For training purposes, three "ideal" samples were selected, each of which likely contained a reasonably high number of sperm cells. For testing following our departure, an additional 10 "challenging" samples were chosen, which contained either a low total number of sperm cells, or relatively few sperm cells compared to the number of female epithelial cells.

The results from the training were successful capture of sperm cells from the non-challenging samples, resulting in full male STR profiles that matched the result obtained by conventional differential extraction (Figure 6-1). However, when the system was tested with 'challenging' samples, several issues arose. It was observed during trapping that large portions of the sperm cell aggregate were lost to the waste fraction, i.e., stabilization of the trapped cluster of sperm cells could not be maintained. It is noteworthy that this occurred after successful bead sweeping to identify the optimal trapping frequency for that chip, but tuning the system to that optimal trapping frequency was not sufficient to maintain stable trapping with the sample. The result of these failed trapping runs were STR profiles that were either partial male profiles, or entirely female due to lack of sperm cells captured (Figure 6-2).

Additionally, it was observed that qPCR data for most post-SONIC samples showed high levels of inhibition of the internal positive control (IPC), indicating that some component of the SONIC

chemistry is detrimental to the PCR kit (Figure 6-3). This was unexpected, as all previous work at UVA had not displayed inhibition of DNA amplification.

THE POTENTIAL BASIS FOR POOR SPERM CELL TRAPPING

We had a strong suspicion that this unwanted effect resulted from an abnormally high number of epithelial cells, and that this translated into a post-lysis sample of abnormally high viscosity. The equation below was discussed on page 9 of this technical report where F_r is the trapping force exerted on a particle:

$$F_r = -\left(\frac{\pi p_0^2 V_c \beta_w}{2\lambda}\right)\cdot\phi(\beta,\rho)\cdot\sin(2kx)$$

$$\phi(\beta,\rho) = \frac{5\rho_c - 2\rho_w}{2\rho_c + \rho_w} - \frac{\beta_c}{\beta_w}$$

where the parameters include P_0 (applied acoustic pressure amplitude), V_c (volume of the particle, i.e., size), β_w (compressibility of the surrounding fluid), λ (acoustic standing wavelength), k (wavenumber defined by $2\pi/\lambda$), x (distance from a pressure node). In the lower equation, an expanded representation of $\boldsymbol{\phi}$, the 'acoustic contrast factor', is given, and it is defined by ρ_c and β_c (density and compressibility of the particle), as well as ρ_w and β_w (density and compressibility of the surrounding medium). Note that parameters describing the surrounding medium show up in two places in force equation (red arrows) – directly as β_w and β_c/β_w as in the $\boldsymbol{\phi}$ term. In other words, density and compressibility of the surrounding medium, i.e., the viscosity of the fluid in which the cells are to be trapped, plays a significant role in the trapping efficiency.

Since bead sweeping to identify the optimal trapping frequency for a particular chip is done on regular buffer, differences in viscosity between the bead sweeping solution (used to identify the optimal trapping frequency) and the sample containing lysed epithelial cells and intact sperm cells, would be largely dependent on the number of cells present. For example, in samples with 1000 sperm cells and the remnants of 1000 lysed epithelial cells, the change in viscosity in a 60 uL volume would not be significant. However, in a sample having a 1:250 sperm cell to epithelial cell ratio, the cellular components of 250,000 lysed epithelial cells could lead to a dramatically higher solution viscosity, one that could shift the optimal frequency needed for trapping to a value substantially different than what was calculated for the chip.

With this hypothesis in mind, and a potential short-term solution to the problem by adjusting the bead sweeping solution, we were fortunate to have the opportunity to address this during follow-up testing at PBSO (detailed later).

THE POTENTIAL BASIS FOR UNEXPECTED INHIBITION

The STR kit defined in-house for protocol development was a custom 5-locus chemistry (AMEL, D2, D8, D12, & D21 loci) that we have termed "faSTPLEX", and the commercial kits used in forensic labs obviously have a larger number of loci, some of which may have been more prone to inhibition. Regardless of this, there was clearly a chemical component of the SONIC chemistry that seemed incompatible with the DNA amplification performed at PBSO (Figure 6-3). We addressed this issue in the short-term by having PBSO analysts remove or reduce the [sarkosyl] used for cell lysis (discussed in detail below), but a permanent solution will need to be found. We are currently working

to address this issue by swapping or removing certain compounds from our cell lysis reagents, and testing the effect on PCR inhibition.

FOLLOW-UP TESTING AT PBSO

The Crouse-Sikorsky team agreed to conduct a second round of SONIC testing in August 2017, contingent on changes being made to the protocol to increase the likelihood of successful trapping of sperm cells.

Inhibition of PCR by SONIC Reagents

In order to determine the source of PCR inhibition observed in earlier testing, samples were prepared with 2800M purified DNA and combinations of our different reagents. The lysis detergent, sperm lysis reagent and buffer, and epithelial lysis reagents were all added to DNA samples and amplified via qPCR to measure any inhibition. Based on the melt curves of the internal positive control (IPC) from the *Promega PowerQuant* kit, the most inhibition was observed in samples containing sarkosyl, a detergent that we use to assure solubilization of cell membranes and other cellular debris. Based on these results, the concentration of sarkosyl in the initial lysis solutions was reduced to from 0.60% to 0.21%. The basis for this was our recognition that sarkosyl is required for full digestion of epithelial cells, but was inhibiting PCR at the concentration of 0.6%. Reducing the concentration by 66% showed lower inhibition when tested with the *Promega PowerQuant* kit, and still assisted in epithelial cell lysis.

Adjusting the Bead Solution Used for Frequency Sweeping

As described earlier, one issue observed during initial testing was the loss of cell aggregates during the trapping stage, even after successful bead trapping was observed in the frequency sweeping protocol. We hypothesized that this was due to physical differences between the bead sweep solution (water, fluorescent beads) and the sample matrix (water, reagents, buffer, cell lysate, intact cells). Those differences could result in changes in fluid viscosity, density, and compressibility, all of which would impact the optimal trapping frequency for particle retention (see earlier discussion on page 9, section 2C). To account for this, we changed the initial lysis step to include a change in volume from 80 uL to 190 uL, leaving 110 uL of cell lysate beyond the required 80 µL for sample separation. This additional remaining cell lysate was used to create what we refer to as a "SURROUNDING MEDIUM (SM)" solution, to which fluorescent beads were added to create an SM solution for frequency sweeping. With this, the instrument scans a range of acoustic frequencies in a solution (SM) that more realistically approaches that of the sample, hence, identifying the optimal frequency for bead trapping with a 'better mimic' of the sample. With this change, we observed a more efficient retention of the sperm cell aggregate during sample trapping in all 6 of the new samples tested; this indicated that the augmented protocol for identifying the optimal trapping frequency successfully addressed the fluidic problems encountered. Figure 6-6 shows the trapping site before and during trapping, as well as during the wash stage post-trapping.

Time-point Collection of Post-Coital Samples

One set of available samples was a time interval study, where post-coital vaginal swabs were collected from the same donor at time points of 5 hours, 24 hours, 48 hours, and 72 hours post-coitus. Prior to SONIC processing, all samples were tested with qPCR to determine initial DNA concentration, and this showed that all samples had excess female DNA with female:male DNA ratios

from 4X to 580X. These samples were tested using the augmented protocol described above, including initial epithelial cell lysis, frequency sweeping with the SM solution, sample trapping and washing, followed by sperm cell extraction and DNA amplification. Figure 6-7 shows the STR profile obtained from the 5-hour post-coital sample, which resulted in a male/female mixed STR profile, where all male alleles are seen, but in the presence of female alleles. This result was unexpected, because videos showed the trapping and washing steps were executed as expected with very efficient trapping of large aggregates of sperm cells.

When testing the 24-hour sample, the PBSO analysts encountered hardware malfunctions. After successful trapping of a sperm aggregate, the washing stage malfunctioned, and pushed air through the chip rather than washing buffer. This resulted in loss of the aggregate to the waste reservoir, and no liquid in the sperm fraction. We were unable to determine the cause of this issue, and it also appeared in subsequent testing. To prevent total loss of sperm aggregate in other samples, the wash step was eliminated. While not ideal, this did allow for processing of the 48 and 72 hour samples without washing. Both samples resulted in female STR profiles in the sperm fraction, believed to be due to lack of washing so that free female DNA was not removed. Upon further analysis of the system when it was returned to UVA, the flow control issues likely stemmed from fluidic connections that had become loose during shipping – the systems WERE NOT engineered to withstand substantial shock and vibration.

Sperm Cell Dilution Samples

Additional samples tested were sperm dilutions ranging from neat semen down to 1:1024 dilutions. The samples were deposited onto green polo and denim fabrics by PBSO analysts. These samples were of particular interest, as they could provide some insight into whether dyes in the fabric would impact either the separation of cells or amplification of DNA. Both samples were trapped effectively via SONIC, and the resulting profiles were entirely male, indicating neither of the common sample substrates negatively impacted SONIC separation (Figure 6-8).

TESTING AT THE MESA POLICE DEPARTMENT

Dr. Kim Meza organized the testing at Mesa PD, which was carried out by Lisa Perry and overseen by Krista Placko. The evaluation dates were February 2nd-February 4th. During analyst training, we tested mock samples comprised of semen deposited onto several materials including nylon and cotton clothing. The Mesa lab asked to blend our technique with their in-house protocols, so initial cell recovery from the sample and post-SONIC extraction was performed with Mesa chemistry. After initial hardware setbacks, successful sperm cell trapping was achieved, but clean male DNA profiles were not obtained from the sperm fraction. The analyst questioned whether the samples might have been inadvertently mixed up or even not extracted at all, as we did observe a mixed male profile from the non-sperm fraction of each sample, as well as complete failure of DNA amplification in the male fraction of mock samples (Figure 6-4). An alternate explanation was hardware failure, as previous valving issues had caused fluid movement to incorrect reservoirs, and could have resulted in mixing of sperm and non-sperm fractions following trapping.

Additional testing was carried out by Lisa Perry in the weeks following on-site training. Mock samples were created using sperm dilutions of 1:10, 1:100, 1:1000, and 1:10,000 of sperm to female epithelial cells (buccal swab source). These samples were prepared in duplicate, one set was run with Mesa chemistry and the other using the SONIC chemistry. The Mesa sample set yielded quality DNA

amplification (indicated by positive controls), but no profiles (Figure 6-5). However, the analyst noted that these samples were not extracted following SONIC separation, indicating that male DNA was not released from the sperm cells, hence, no profile could be expected. All of the mock samples tested using the SONIC protocol showed PCR inhibition, similar to what was observed at PBSO testing. This reinforced the possibility that a chemical component of the SONIC chemistry was incompatible with PCR kits used in state forensic labs.

OVERVIEW AND FEEDBACK

The opportunity to test our prototype instruments at the Palm Beach and Mesa labs was crucial to our being able to put some quantitative value on the potential use of the SONIC technology in forensic labs. Analysts at both locations were intrigued by the ADE concept, enthusiastic about its potential application, and believed that this technology could have a significant impact on their workflow. We are grateful The SONIC system was capable of capturing sperm cells from real casework samples that had been kept in storage, and produced DNA profiles matching the result from lab-specific conventional differential extraction. We encountered setbacks with testing of more challenging samples, which resulted in loss of trapping ability and failure to generate clean male DNA profiles.

I want to emphasize how incredibly fortunate we were to have the evaluation support from both the PBSO and Mesa PD. These directors and their staff are slammed constantly with casework, and yet they have the drive to contribute to (potentially) new technology, despite complicating or extending their work day.

The feedback given from PBSO analysts can be summarized as follows. They seem to believe that the SONIC technology is a fascinating potential avenue for improving differential extraction and, in the opinion of the analysts, if this development were successful, it would not be a high bar to get other analysts and labs on-board for trial the SONIC system. After two full rounds of prototype testing, PBSO indicated they would happily continue evaluating the system and provided feedback, on the condition that we keep working to address any shortcomings and update the hardware to deal with problematic samples. Comments from those at the director level follow.

Dr. Cecelia Crouse at PBSO

"The prototype SONIC instrument was compact, intuitive to use, and provides a new approach for differential extraction. We were impressed with the technology's ability to capture sperm from exemplary sexual assault samples, and with some adjustments to hardware and chemical processes, we believe that this system could also move to address more challenging samples (such as those with low total sperm or excess epithelial cells). We would support the further development and testing of this technology, and would be excited to field-test a second-generation system with the necessary improvements."

Dr. Kim Meza at Mesa PD

"*We tested the SONIC system on a variety of mock sexual assault samples, and our in-house analyst worked first-hand with the prototype. The technology was exciting to use, but we encountered some difficulties due to damage to the instrument during shipping. With the necessary repairs made, we were able to capture sperm cells from some samples, and the protocol removed several sample handling steps from the normal process. Several changes are required to make this technology a viable supplement to conventional differential extraction, namely more robust hardware and*

complementary chemical adjustments, but we would be in favor of more development and testing of the SONIC systems."

MERITS AND DEMERITS OF THE SONIC TECHNOLOGY

There were several positive outcomes with this technology development adventure. Even more so than we did when we wrote this proposal, we believe this technology can be developed successfully, and has a place in forensic DNA analysis. Its MERITS included:

- that it is a compact hardware that can effectively trap sperm cells in crude mixtures, and that this could supplant several steps associated with conventional differential extraction
- that the system is highly automated, with a simple use GUI, and, collectively, substantially reduces pipetting/washing steps for each sample
- reducing the sample processing time, from sample to PCR-ready DNA, to less than 1 hour
- very positive responses from the forensic analysts, who are enamored by the potential for the technology, and would be receptive to testing updated instrumentation

However, as with the research and development of any technology, it is not without demerits. Through discussions with those who have trialed the technology, these included:

- that it has not been optimized for use with multiple commercial forensic chemistries
- that it cannot currently handle samples with an excess of epithelial cells
- that the prototypes are not robust enough for extensive shipping
- that the current system can handle a single sample at a time (although this was described as a 'nice-to-have', not a 'must-have')

THE FUTURE POTENTIAL FOR SONIC TECHNOLOGY

Potential for Impact

We believe the potential impact of this technology on the forensic field is three-fold: 1) **Analysts** will have less hands-on time per sample, allowing them to focus on STR mixture analysis and sample preparation for a more efficient process. They will also no longer rely on personal pipetting skill to determine the quality of separation, as the automated process would create reproducibility independent of analyst. 2) **Labs** will reduce sample backlogs and more easily keep up with incoming samples. 3) **Criminal Justice** will be enhanced by faster investigation resolution, and the testing of a greater number of potentially incriminating samples.

Problems Encountered and Imminent Solutions

There were three unexpected issues encountered that surprised us, and, in retrospect, both issues fall neatly into a category that could be entitled *"why forensic samples are the most challenging analytical chemists might encounter"*. These were:

1) Problems with the frequency sweep approach to defining the optimal trapping frequency.
2) Unanticipated bandwidth of sample cell numbers
3) The chemistry we developed for 'pre-SONIC' processing presents inhibition with some kits.

<u>The Frequency Sweep Problem</u> - we (apparently, naively) assumed that the number of cells involved (both male and female combined) would be adequately diluted in the pre-SONIC processing in the

vast majority of the cases. This turns out to NOT be the case. There is substantial variation in the total number of cells obtained from casework samples – ranging from tens of cells up to several hundreds of thousands. The impact of this is that our initial frequency sweep with fluorescent beads identified a trapping frequency that was non-optimal for a high cell count sample, and resulted in poor sperm cell trapping. As described above (section x), we believe that cell lysis results in a substantially increased solution viscosity. This problem can be solved with some engineering and redesign of the chip.

This can be corrected by approaches described by Nilsson et al. (*Lab on a Chip*, 2014) (for another application) where they constantly tune the optimal frequency in response to changes in the sample. By measuring 'electrical impedance' of the solution in real-time during trapping, and feeding that information back to the frequency generator, we will be able to change the optimal frequency as the characteristics of the surrounding medium changes, and on the millisecond timescale. This is tantamount to cruise control on a car, where the throttle changes as needed to maintain a steady speed. This will allow for the frequency to be changed 100's of times per second to account for changes in viscosity. This approach has not been demonstrated for sperm cell trapping but should be feasible. With some re-engineering of the hardware, some redesign of the chip and some research effort, we are confident that this could be implemented in future iterations of the SONIC technology.

The Sample Bandwidth Problem – In order to have an idea of what to expect with forensic sexual assault samples, we created a questionnaire that was sent to the directors of a select number of laboratories. This is found in Appendix 6. Even given the responses from a number of labs, what never anticipated that the male-female cell number ration would range over five orders of magnitude – M/F ratio from 100:1 to 1:1000. This is directly linked to the first issue (above) and, therefore, **this will be corrected by** the impedance-driven frequency tuning changes described above.

The Inhibition Problem - The final problem we encountered was a reagent independence issue. Since each forensic lab defines their own differential extraction protocol, there is no uniform set of reagents used nationwide. As a result, we used a chemistry optimized in-house that did not translate well to the PBSO or Mesa PD labs, as both experienced PCR inhibition with the extracted sperm cells. **This can be corrected** in the short-term, by reducing the sarkosyl concentration in the initial cell lysis – this appears to have resolved this issue. **This can be corrected** with a longer-term approach, but this requires additional research time and effort; given that, we would have a chemistry that ultimately allows for optimal cell lysis, extraction, and PCR steps with any samples, and compatible with the full 23-loci kits available to forensic labs.

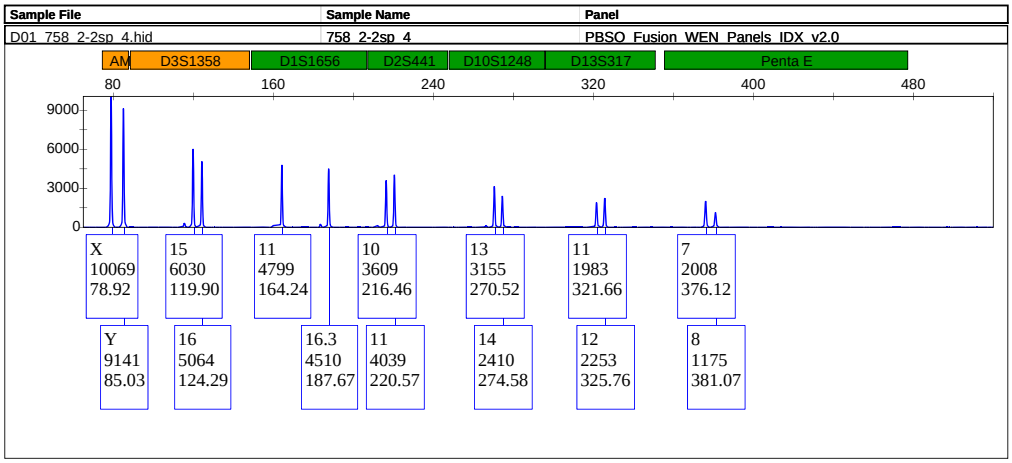
SUMMARY

We believe that remarkable progress was made towards addressing the difficult and time-consuming process of differential extraction. The acoustic trapping technology inherent with the SONIC instrumentation has the potential to change sexual assault investigation for the analysts, labs, and victims. The only issue that remains, and deserves discussion with the NIJ, is whether the progress made to-date warrants follow-on funding support the remaining research and development needed to push this project through its current "early prototype" phase, to a more ruggedized instrument and chemistry that, with positive evaluation, could be commercialized.

FIGURES – EXTERNAL EVALUATION

Figure 6-1 - Male STR profile from ideal sexual assault samples

The following STR profiles were obtained from a sheet cutting that had a high amount of visible sperm under microscopy. This resulted in successful trapping of sperm cells by acoustic capture, and after DNA extraction there was enough male DNA present to generate an STR profile that is entirely male, and matches the profile obtained by PBSO through conventional differential extraction.



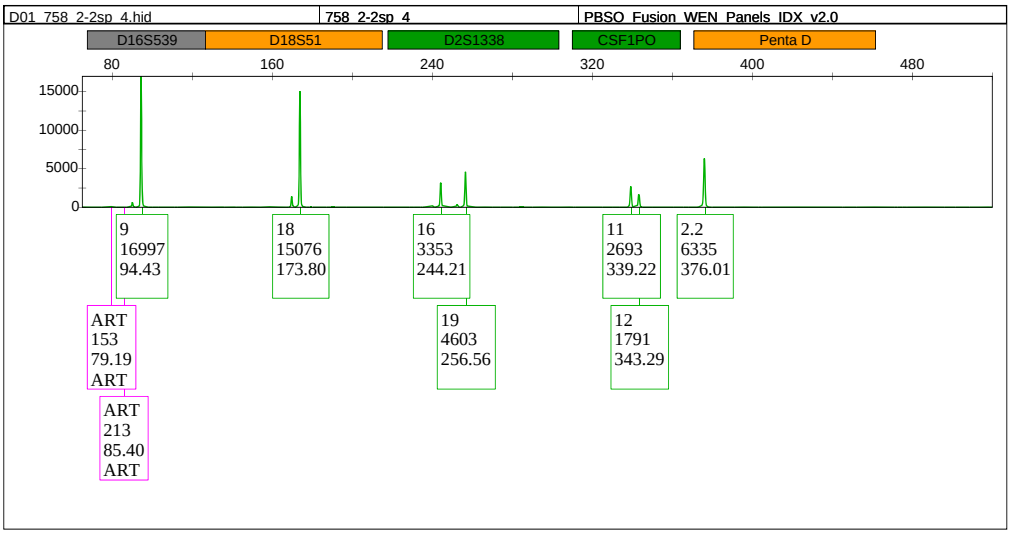


Figure 6-2 - Sperm and non-sperm profiles from challenging STR samples

a. The first profile shown is the non-sperm fraction from a rectal swab. The profile is incomplete, with several absent loci. The peaks that are present are predominantly female.

b. The profiles below are from the sperm fraction of the same rectal swab sample. Some trapping was observed during SONIC acoustic capture, but notn enough sperm were retained to generate a full profile. The resulting STR profile is mixed, with some male and female peaks present.

Figure 6-3 - qPCR table from PBSO

This table was assembled from qPCR data over dozens of runs with real samples conducted at PBSO. One major conclusion drawn from this data is that part of the SONIC chemistry is inhibiting PCR. The IPC threshold flags as inhibited only when SONIC chemistry is used, as opposed to the PBSO chemistry in conjunction with SONIC separation.

Palm Beach County Sheriff's Office - Promega PowerQuant® System
Combined Results Table for SONIC Project

	Sample Name	[Auto]	[Deg]	[Y]	Auto Cq	IPC Cq	Y Cq	Deg Cq	Closest IPC	IPC Shift	IPC Threshold	[Auto]/[Y]	[Auto]/[Y] Threshold	[Auto]/[D]	[Auto]/[D] Threshold
01-30-17 SONIC Training	412_1Ans_1	33.6927	4.7227	0.2371	22.085	21.815	28.686	24.547	30.218	-8.403	Below	142.104	At or Above	7.134	At or Above
	412_1Asp_2	2.0900	0.2762	0.0970	26.0031	24.6604	29.9655	28.7849	22.3204	2.3400	At or Above	21.5548	At or Above	7.5673	At or Above
	758_2-2ns_3	13.5169	4.1697	9.3002	23.3720	21.7955	23.4366	24.7332	22.3204	-0.5249	Below	1.4534	Below	3.2417	At or Above
	758_2-2sp_4	39.8539	7.1146	58.5886	21.8479	Undetermined	20.8034	23.9356	30.2181		At or Above	0.6802	Below	5.6017	At or Above
	844_1-9ns_5	0.7989	0.1329	0.2615	27.3585	21.2905	28.5460	29.8772	22.9501	-1.6596	Below	3.0550	At or Above	6.0136	At or Above
	844_1-9sp_6	0.1243	0.0259	0.1209	29.9807	Undetermined	29.6497	32.3170	22.9501		At or Above	1.0281	Below	4.7976	At or Above
01-31-17 SONIC Training	412_1Cns_1	0.1281	0.0032	0.0143	28.923	20.821	31.477	34.059	20.306	0.516	At or Above	8.978	At or Above	40.069	At or Above
	412_1Csp_2	0.0124	0.0008	0.0032	32.2766	20.9426	33.5168	36.0865	20.4707	0.4719	At or Above	3.8127	At or Above	15.9542	At or Above
	844_2C-2ns_3	0.0263	0.0004	0.0012	31.1935	20.5589	34.8580	36.9628	20.4707	0.0882	Below	21.4504	At or Above	62.5470	At or Above
	844_2C-2sp_4	0.0008		0.0001	36.2685	20.6994	38.7282	Undetermined	20.4707	0.2287	Below	10.3666	At or Above	Undetermined	At or Above
02-06-17 Non SONIC - used conventional methods to compare samples to SONIC (age)	412_1Cns_1	0.0258	0.0015	0.0016	31.157	20.467	34.303	35.044	20.469	-0.002	Below	16.169	At or Above	17.521	At or Above
	412_1Csp_2	0.0085	0.0018	0.0052	32.7156	20.5852	32.7197	34.7909	20.4693	0.1160	Below	1.6424	Below	4.8121	At or Above
	844_2C-2ns_3	0.0075	0.0007	0.0008	32.8872	20.3554	35.3178	36.0348	20.4693	-0.1139	Below	9.9633	At or Above	10.2535	At or Above
	844_2C-2sp_4	0.0001		0.0001	38.8389	20.3579	38.4340	Undetermined	20.4693	-0.1114	Below	1.4265	Below	Undetermined	At or Above
	RCNS_5				Undetermined	20.3013	Undetermined	Undetermined	20.4693	-0.1680	Below				
	RCSP_6				Undetermined	20.3791	Undetermined	Undetermined	20.4693	-0.0902	Below				
04-17-17 Used PBSO reagents in combination with SONIC instrument; post-SONIC purification with EZ1	469_3-3ns_1	0.1777	0.0297	0.0369	29.049	20.830	30.760	31.504	20.708	0.123	Below	4.811	At or Above	5.988	At or Above
	469_3-3sp_2	0.0081	0.0017	0.0020	33.4677	20.5968	34.8926	35.6181	20.8600	-0.2632	Below	4.0371	At or Above	4.6574	At or Above
	289_3-1ns_3	1.0290	0.2929	0.1325	26.5354	20.8253	28.9471	28.1822	20.7078	0.1175	Below	7.7650	At or Above	3.5126	At or Above
	289_3-1sp_4	0.0651	0.0205	0.0092	30.4855	20.5765	32.7257	32.0401	20.7078	-0.1312	Below	7.0450	At or Above	3.1765	At or Above
	412_1Ans_5	14.0636	2.0708	0.0235	22.7926	20.5561	31.4034	25.3448	20.7370	-0.1809	Below	599.1305	At or Above	6.7913	At or Above
	412_1Asp_6	0.7458	0.1348	0.0019	26.9960	20.5922	34.9489	29.3081	20.7078	-0.1155	Below	386.3917	At or Above	5.5325	At or Above
	376_14-1ns_7	0.0334	0.0028	0.0001	31.4421	20.8738	38.5895	34.9250	20.8600	0.0138	Below	224.9058	At or Above	11.8926	At or Above
	376_14-1sp_8	0.0023	0.0003		35.2703	21.0098	Undetermined	38.2109	20.8600	0.1497	Below	Undetermined		7.8943	At or Above
	980_6Cns_9	0.4836	0.0879	0.0006	27.616	20.558	36.566	29.928	20.708	-0.150	Below	782.654	At or Above	5.499	At or Above
	980_6Csp_10	0.0729	0.0172	0.0006	30.3235	20.7412	36.5335	32.2964	20.7078	0.0335	Below	115.4203	At or Above	4.2447	At or Above
	RCNS_11				Undetermined	20.4122	Undetermined	Undetermined	20.8600	-0.4478	Below				
	RCSP_12				Undetermined	20.4767	Undetermined	Undetermined	20.8600	-0.3833	Below				
04-19-17 All SONIC	957_5-13ns_1	0.3790	0.0515	0.1852	27.771	20.999	28.386	30.456	20.816	0.182	Below	2.046	At or Above	7.365	At or Above
	957_5-13sp_2	0.0334	0.0034	0.0165	31.1689	21.1259	31.7896	34.3250	20.7112	0.4147	At or Above	2.0227	At or Above	9.8539	At or Above
	844_1-5ns_3	0.0531	0.0153	0.0179	30.5201	20.4359	31.6781	32.1808	20.8161	-0.3802	Below	2.9712	At or Above	3.4705	At or Above
	844_1-5sp_4	0.0014		0.0003	35.6273	20.9902	37.5497	Undetermined	20.7112	0.2790	Below	4.9959	At or Above	Undetermined	At or Above
	980_6Ans_5	2.3423	0.4581	0.0259	25.2222	20.9354	31.1564	27.3455	20.5714	0.3640	At or Above	90.4568	At or Above	5.1132	At or Above
	980_6Asp_6	0.1258	0.0098	0.0014	29.3132	21.6350	35.2956	32.8102	20.8161	0.8189	At or Above	91.8752	At or Above	12.7976	At or Above
	976_1Gns_7	25.3224	1.6384	0.3636	21.8913	21.0277	27.4361	25.5325	20.5714	0.4562	At or Above	69.6473	At or Above	15.4556	At or Above
	976_1Gsp_8	1.7174	0.0551	0.0275	25.6564	22.0097	31.0700	30.3589	20.5714	1.4383	At or Above	62.3771	At or Above	31.1777	At or Above
	839_2B-6ns_9	4.1264	0.0885	0.1678	24.430	20.960	28.525	29.684	20.571	0.389	At or Above	24.587	At or Above	46.624	At or Above
	839_2B-6sp_10	0.2131	0.0103	0.0197	28.5760	21.0218	31.5444	32.7385	20.8161	0.2057	Below	10.8427	At or Above	20.6097	At or Above
	039_1Gns_11	0.0208	0.0003	0.0022	31.8350	20.5095	34.6454	37.9384	20.7112	-0.2018	Below	9.5491	At or Above	77.6139	At or Above
	039_1Gsp_12	0.0009		0.0005	36.2966	21.0298	36.7316	Undetermined	20.7112	0.3186	At or Above	1.7321	Below	Undetermined	At or Above

PBSO = Minimum of 15 pg of DNA (total amount amplified) to proceed to auto STR amp and 17 pg of DNA (total amount amplified) to proceed to Y-STR amp. Blank spaces = negative.

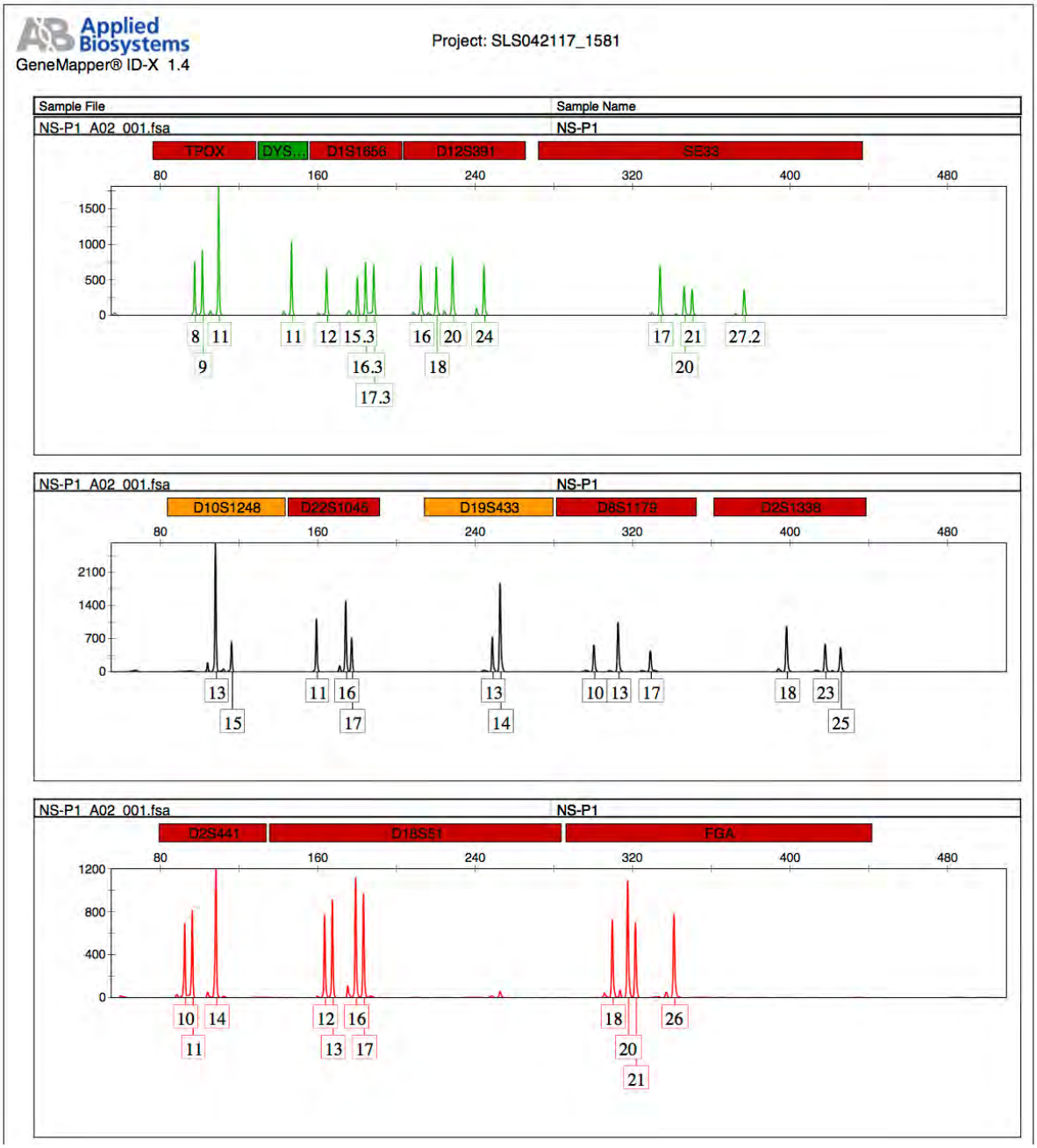
IPC shift = At or Above 0.3, Possible inhibitor present

At or Above 2 indicates possible Male/Female mixture; Auto/Y ratio is ≥ 200 = PBSO direct to Y-STR

Auto/Degradation = At or Above 2.0, Possible degraded sample

Figure 6-4 STR profiles from Mesa

a. Non-sperm fraction, mock sample P1. Below is the STR profile from the non-sperm fraction of a mock sample separated via SONIC. The result is mixed profile, with multiple peaks from both male and female cell donors visible.



b. Sperm fraction, mock sample P1. The STR profile below has no peaks visible at any locus. Normally this would indicate trapping failure, however the analyst at Mesa noted that this

sample was mistakenly not extracted after sperm trapping. This means trapping could have been successful, but the DNA was not liberated.

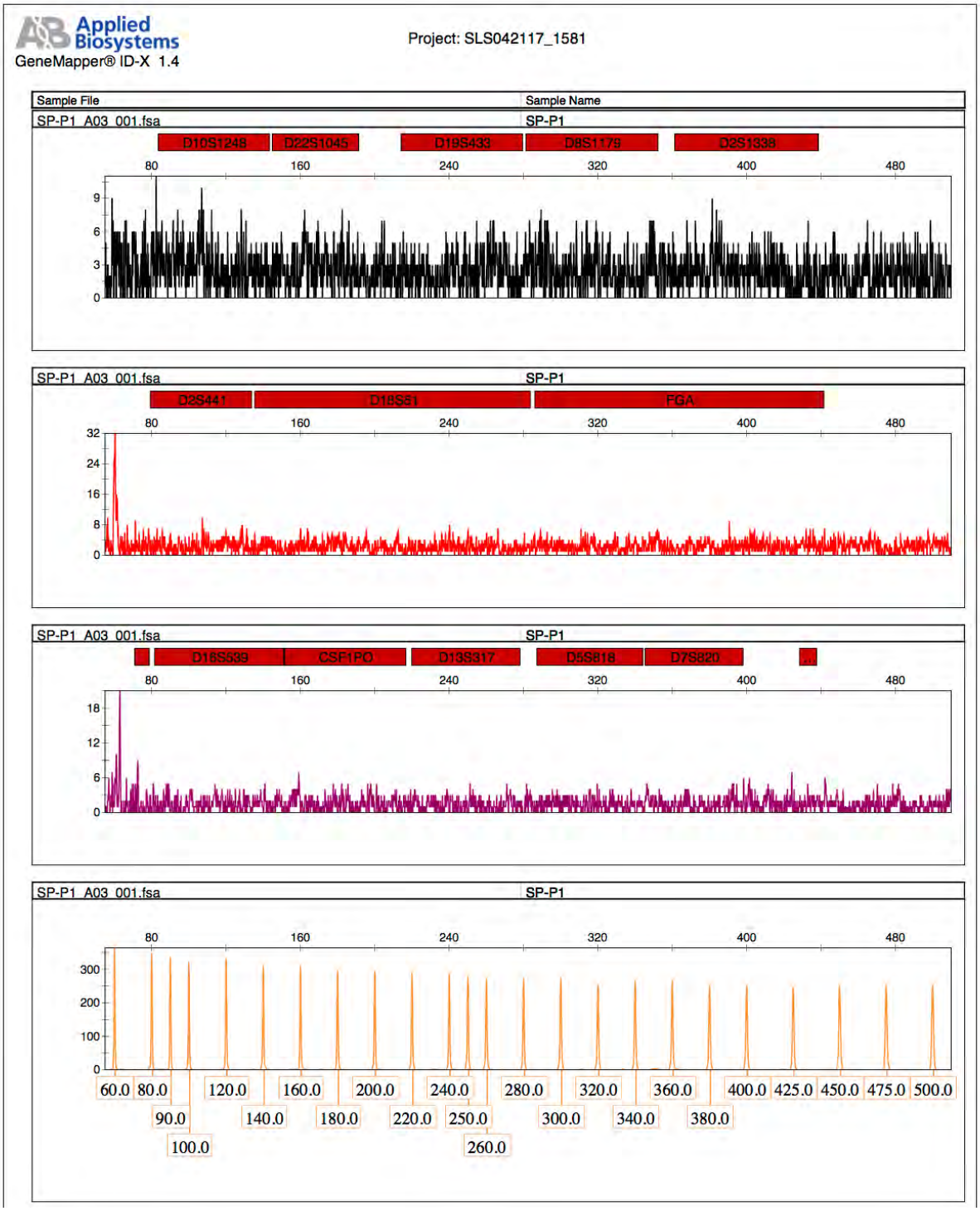
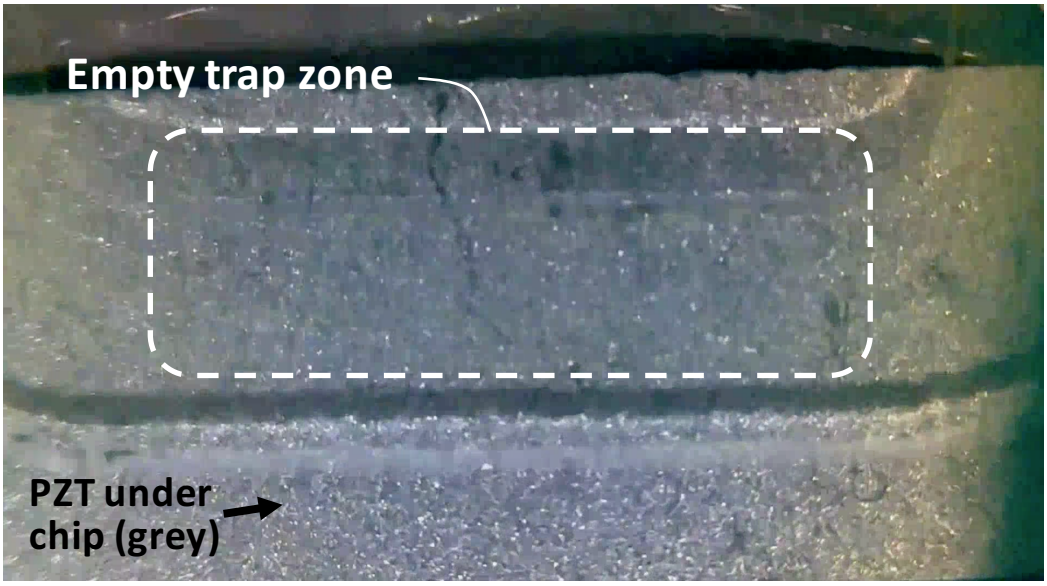


Figure 6-5 Table of Mesa PD results

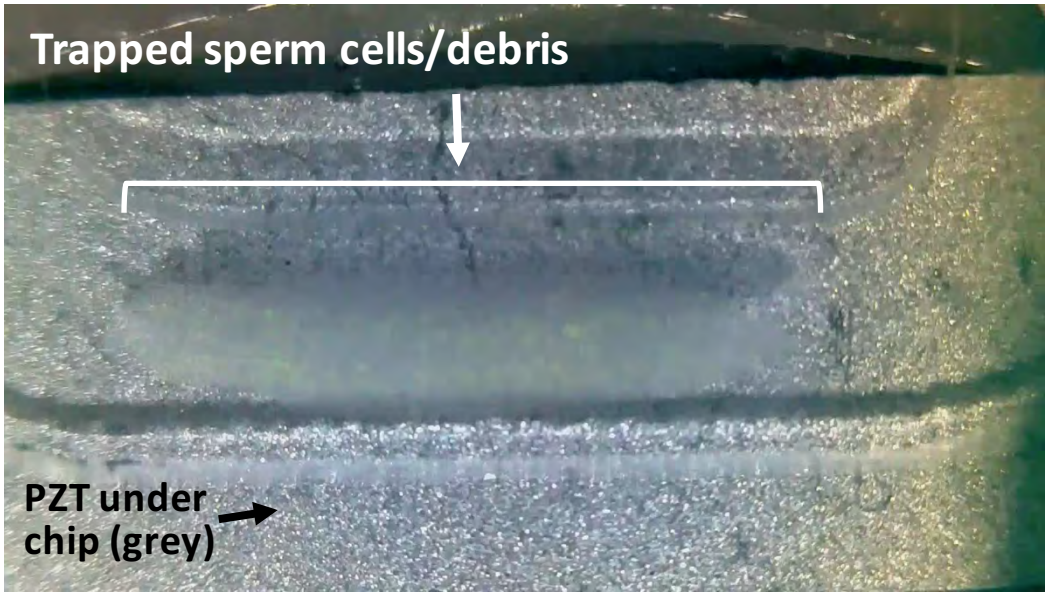
Of the mock samples prepared by Mesa PD, only two (M1 and P4) resulted in any profile obtained. This indicates possible inhibition of PCR as seen by the failed quality sensors, which corroborates the qPCR data from PBSO.

Sample name	Semen dilution	Microscopy results	STR results
M1	1:10	4+ (multiple sperm)	Mixed profile
M2	1:100	2+ (more than 10 sperm)	No profile, quality sensor okay
M3	1:1,000	2+	No profile, quality sensor fail
M4	1:10,000	0 (no sperm)	No profile, quality sensor fail
P1	1:10	NA	No profile, quality sensor fail
P2	1:100	NA	No profile, quality sensor fail
P3	1:1,000	NA	No profile, quality sensor fail
P4	1:10,000	NA	Partial female profile

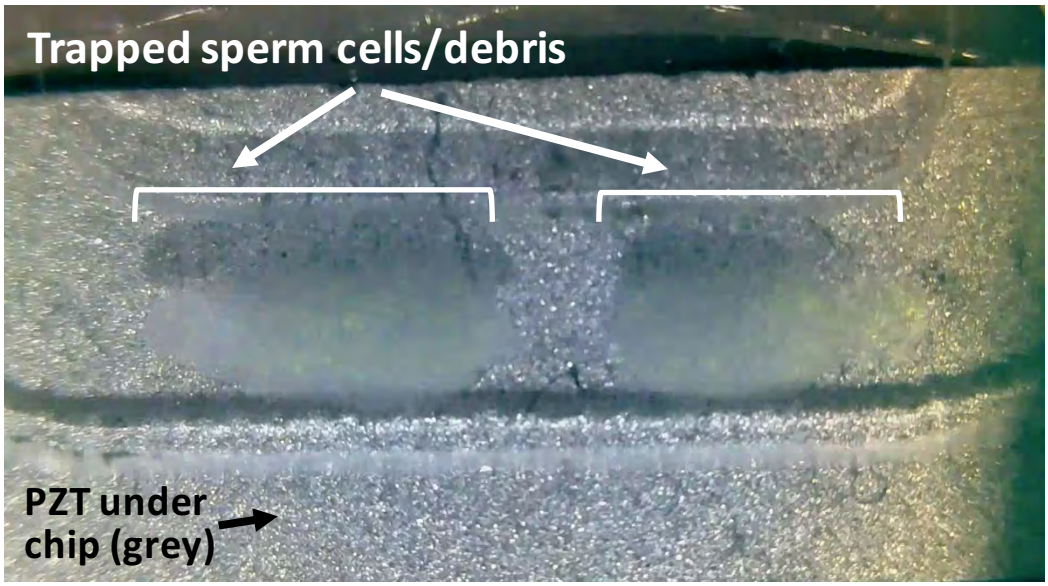
Figure 6-6 - Images of trapped aggregate from August 2017 samples



Pre-trapping: fluidic channel is empty, no aggregate visible.



Sample trapping stage: large aggregate of cells and debris present.



Sample washing stage: debris washed away, leaving smaller cell aggreagtes trapped.

Figure 6-7 – 5 hour time delayed sample.

STR profile is mixed male/female, with dominant female contributing peaks. This indicates that sperm cells were captured, but not effectively purified.

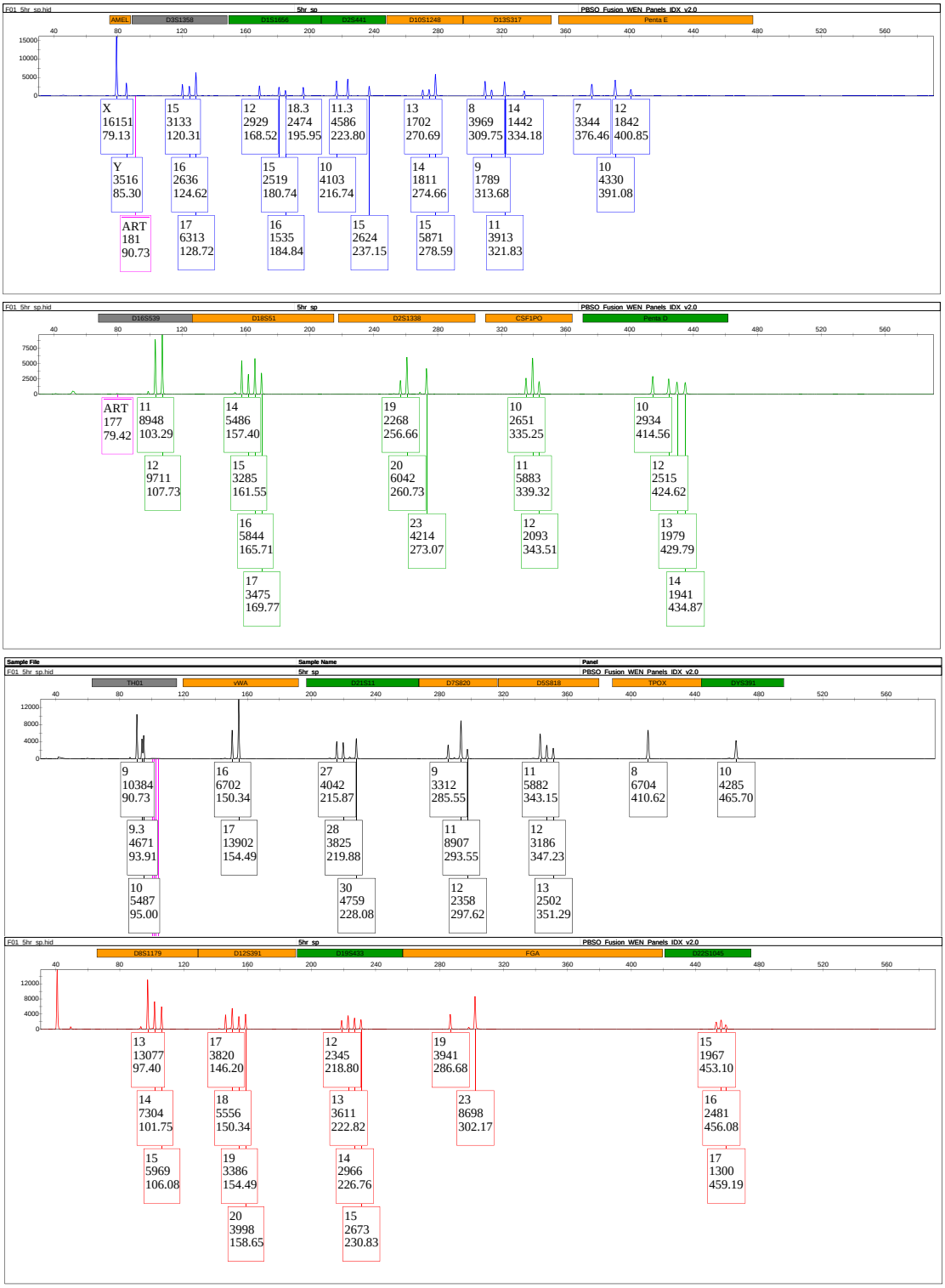
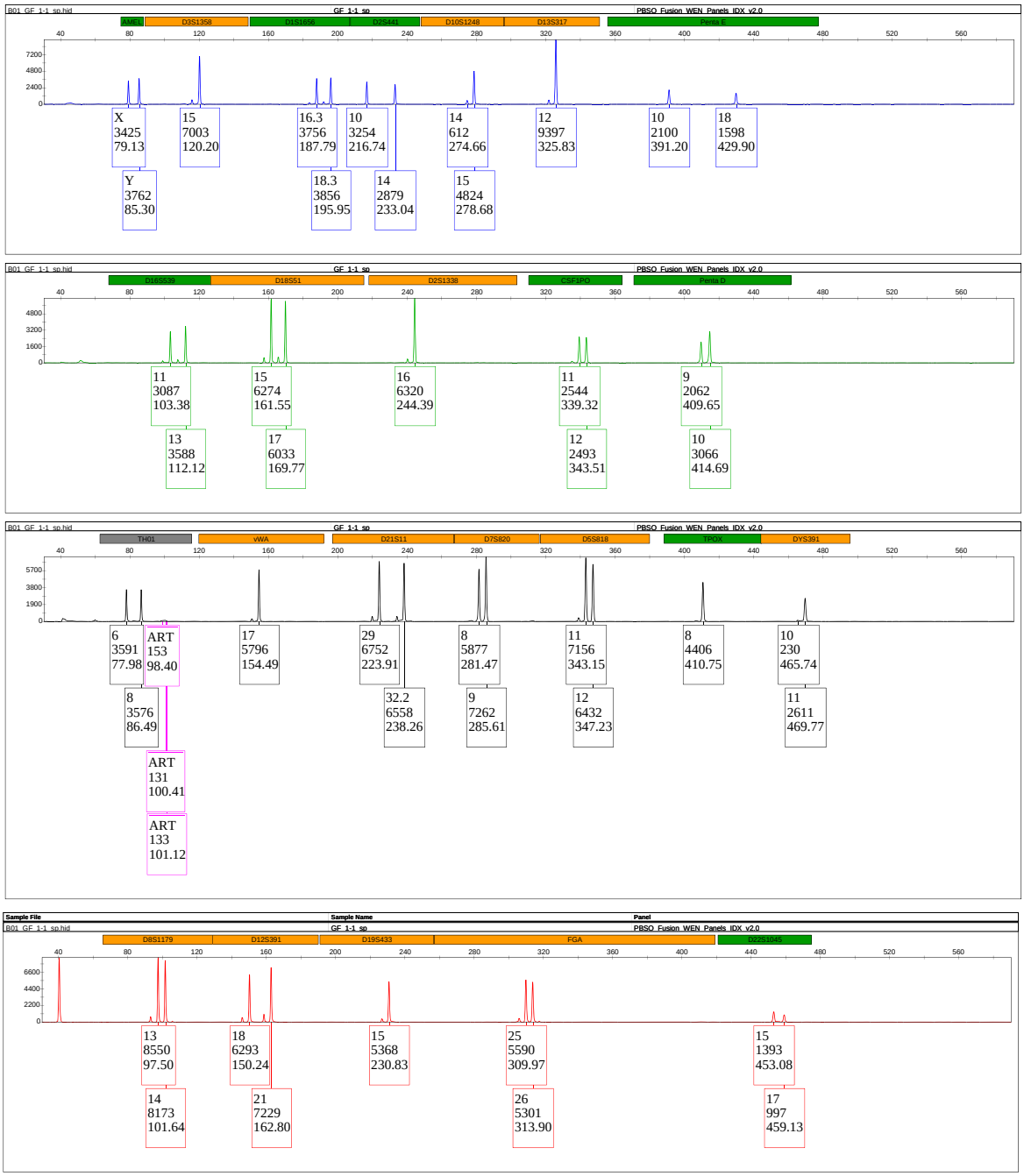


Figure 6-8 - GF dilution sperm fraction

Complete male profile with strong peak heights (>1000 RFU) and good resolution indicates that fabric substrate did not negative impact SONIC trapping of sperm cells.



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APPENDIX 2 - SUPPLEMENTARY FIGURES

Figure S1: Pre-SONIC and post-SONIC profiles of a 4:1 sample, female donor 003. The sperm fraction shows an incomplete male profile, with loss of peaks at heavier loci.

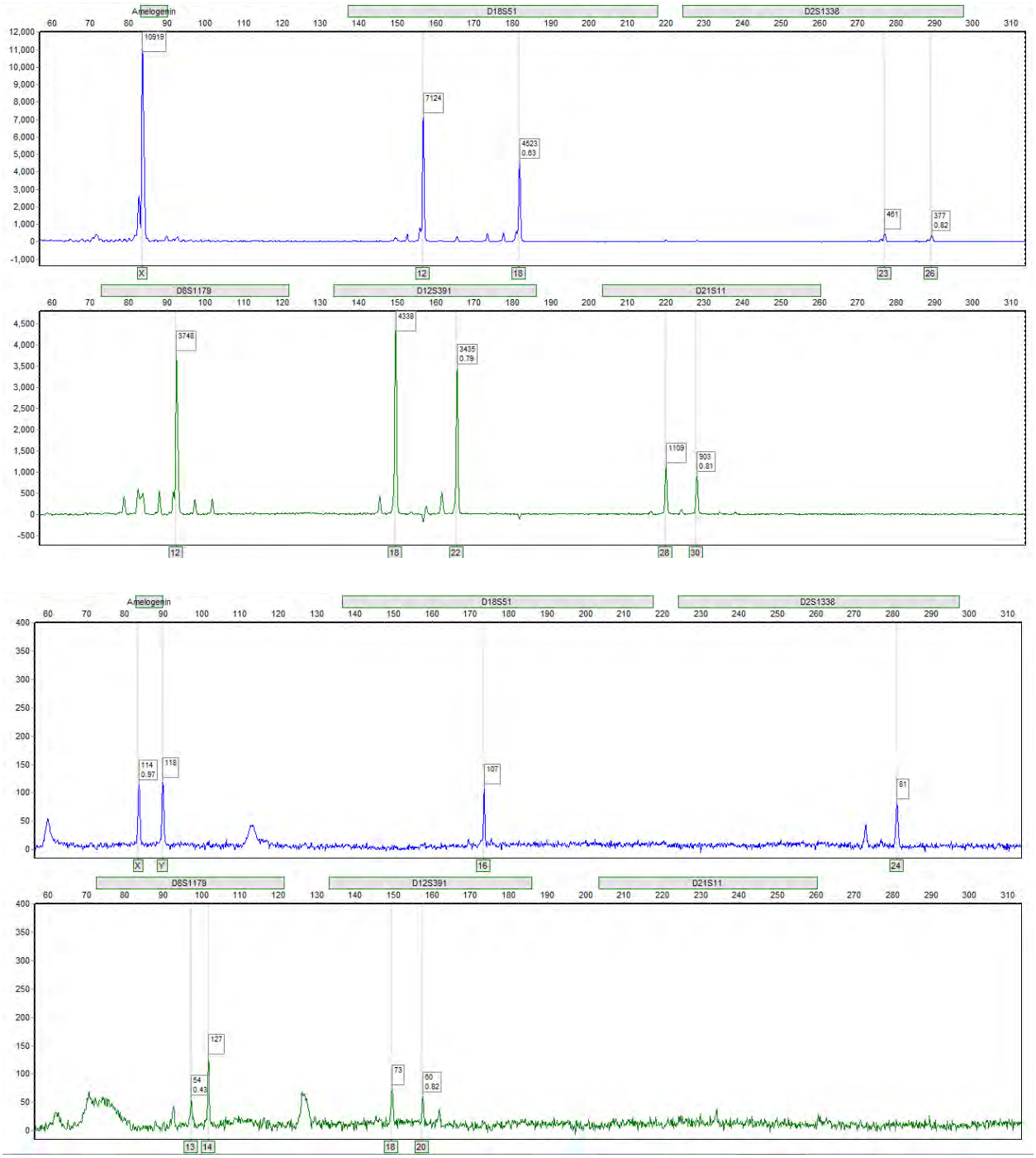


Figure S2: Pre-SONIC and post-SONIC profiles of a 9:1 sample, female donor 004. The sperm fraction shows a partial male profile, lacking the D21 locus.

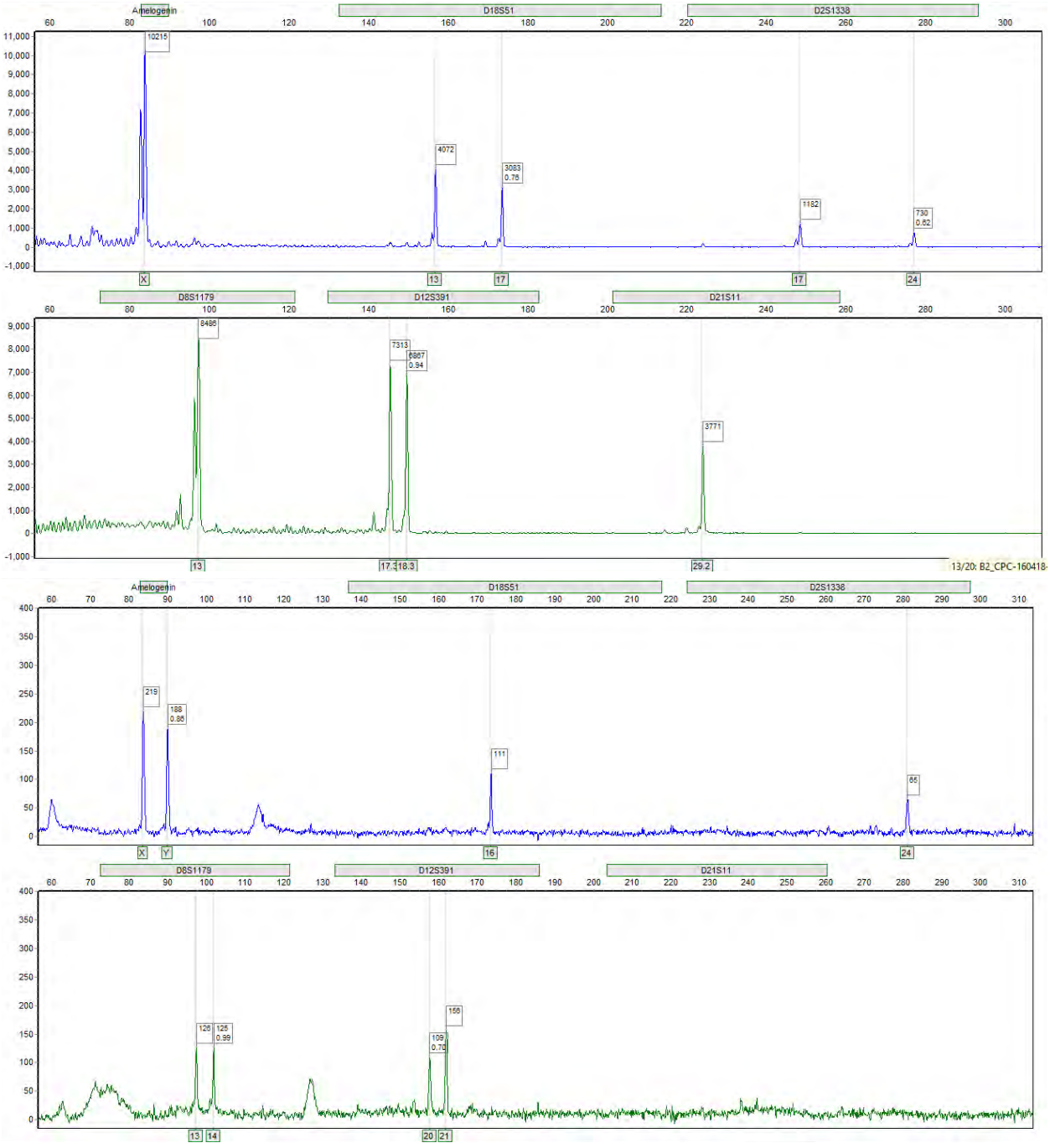


Figure S3: Pre-SONIC and post-SONIC profiles of a 3:1 sample, female donor 006. The mixed profile is entirely female, and from the sperm fraction is obtained a complete male profile with strong peak heights.

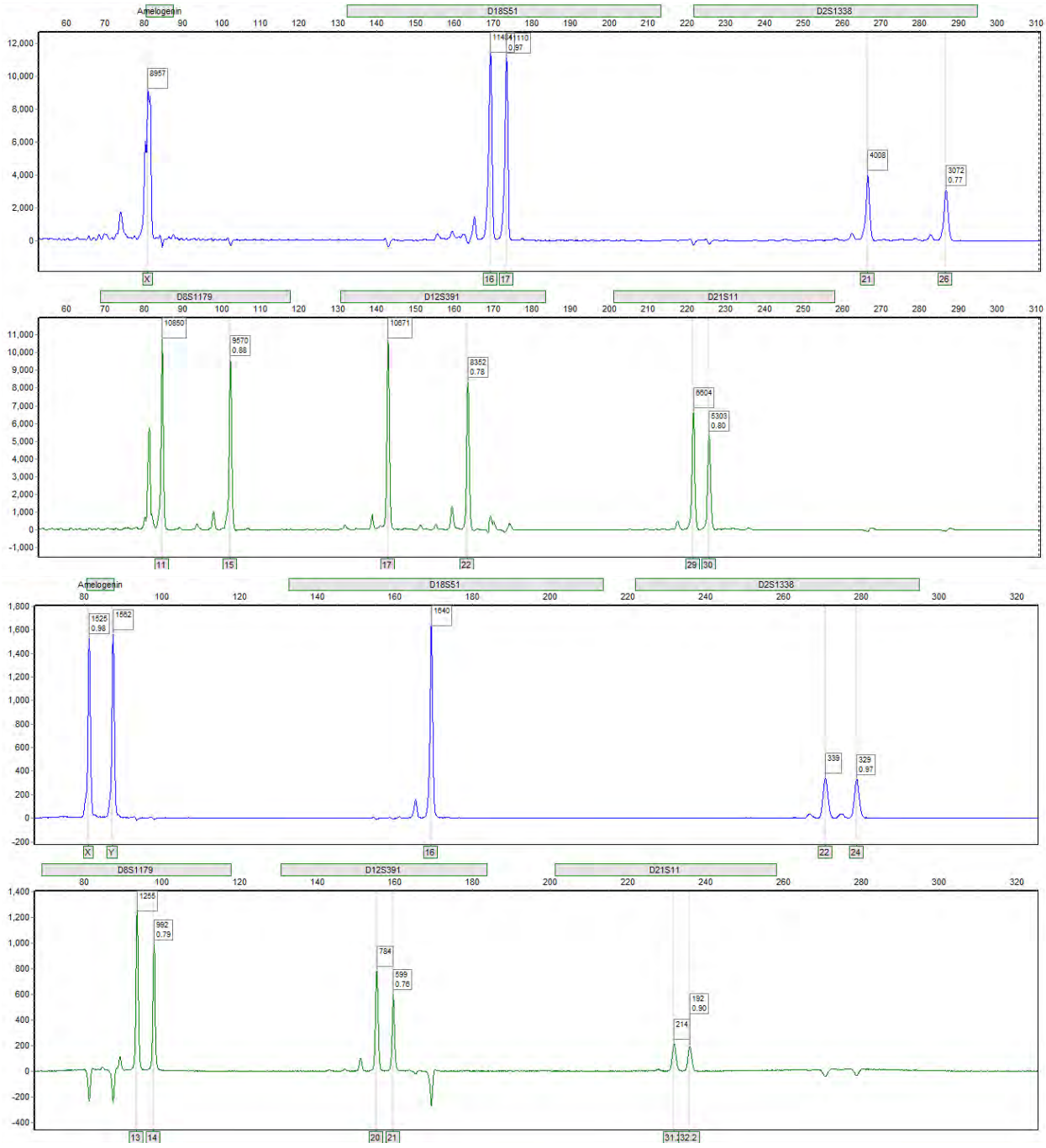


Figure S4: Pre-SONIC and post-SONIC profiles of a 4:1 sample, female donor 007. A complete male profile is obtained from the sperm fraction.

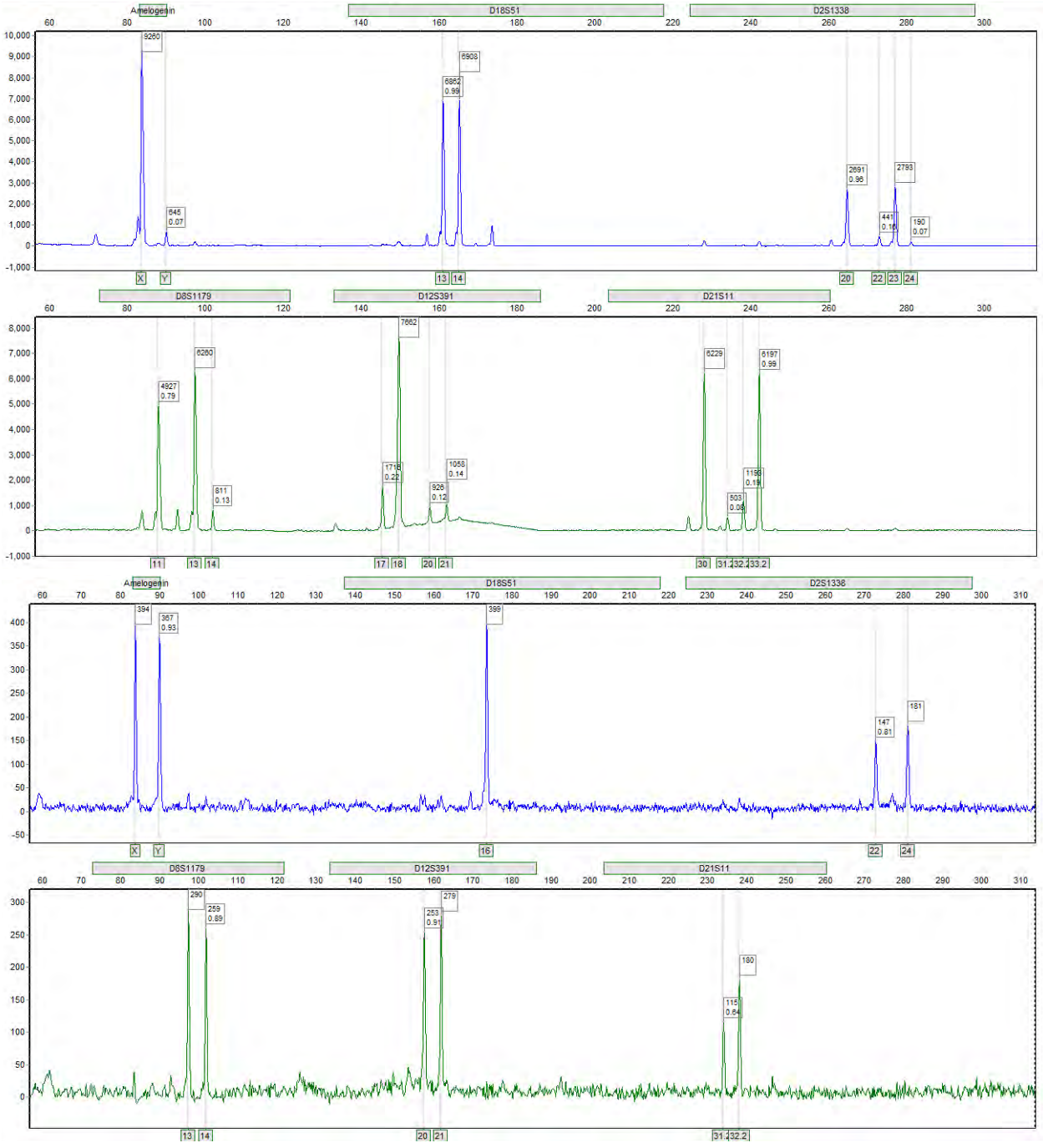


Figure S5: Pre-SONIC and post-SONIC profiles of a 5:1 sample, female donor 010. The sperm fraction profile has low peak heights, but is a full male profile.

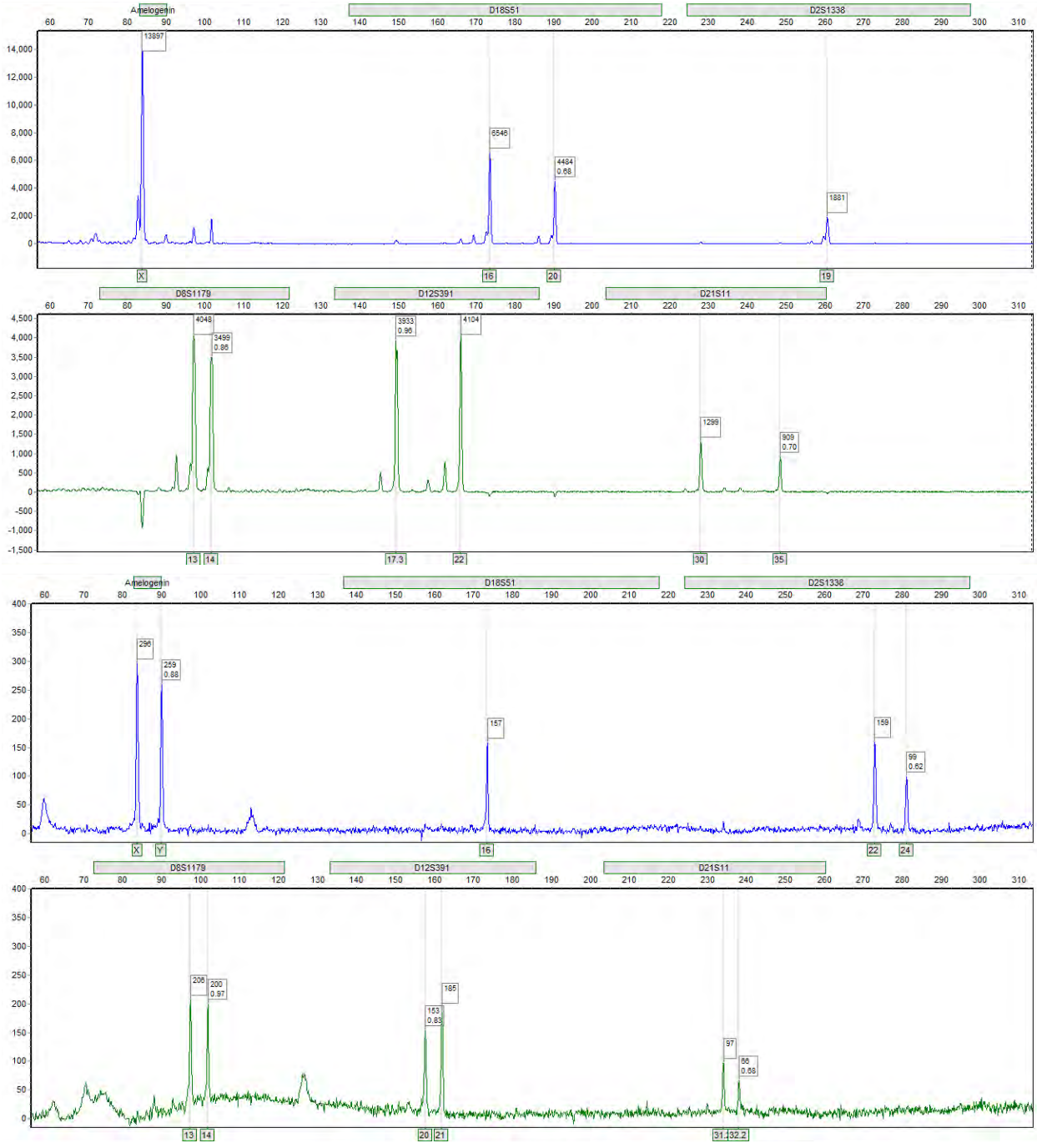


Figure S6: Pre-SONIC and post-SONIC profiles of a 3.5:1 sample, female donor 013. The sperm fraction has a complete male profile with strong peak heights.

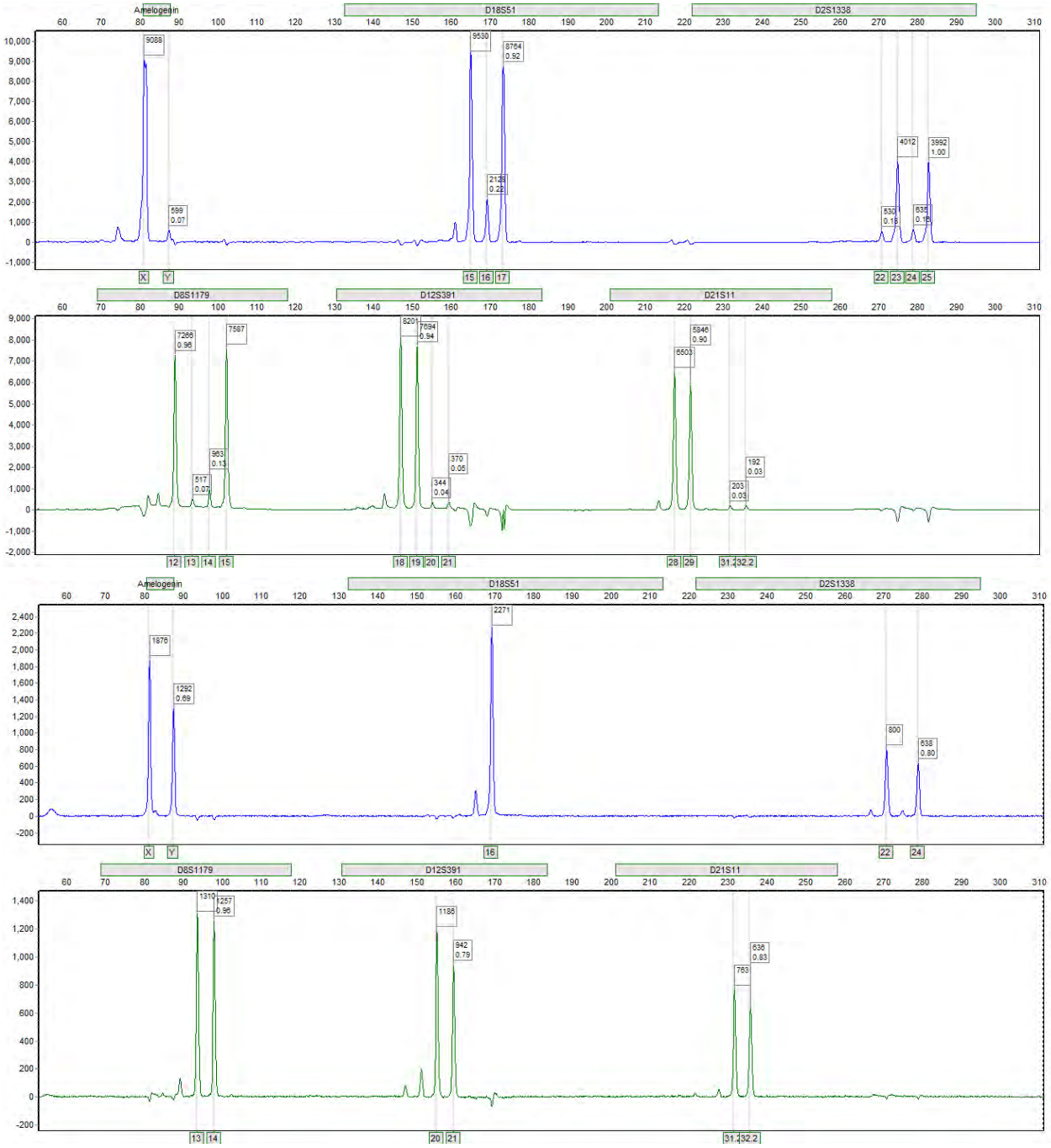


Figure S7: Pre-SONIC and post-SONIC profiles of a 2:1 sample, female donor 015. The profile from the sperm fraction has low peak heights and a slight ski slope effect at heavier loci.

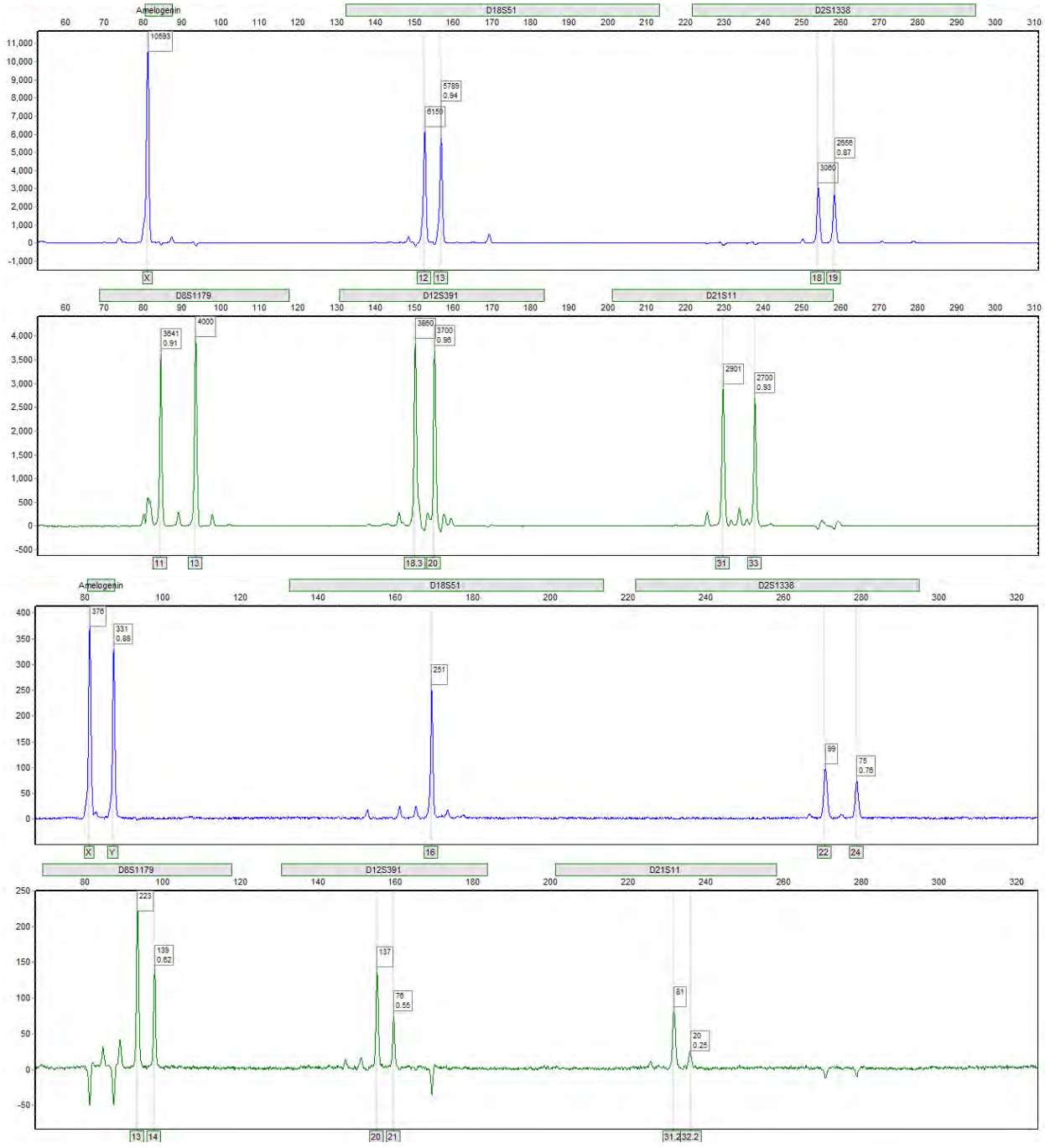


Figure S8: STR profiles from 1,000 sperm cell extraction. The profile from the newer sperm sample (lower) shows over 10X peak heights compared to the old sample (upper).

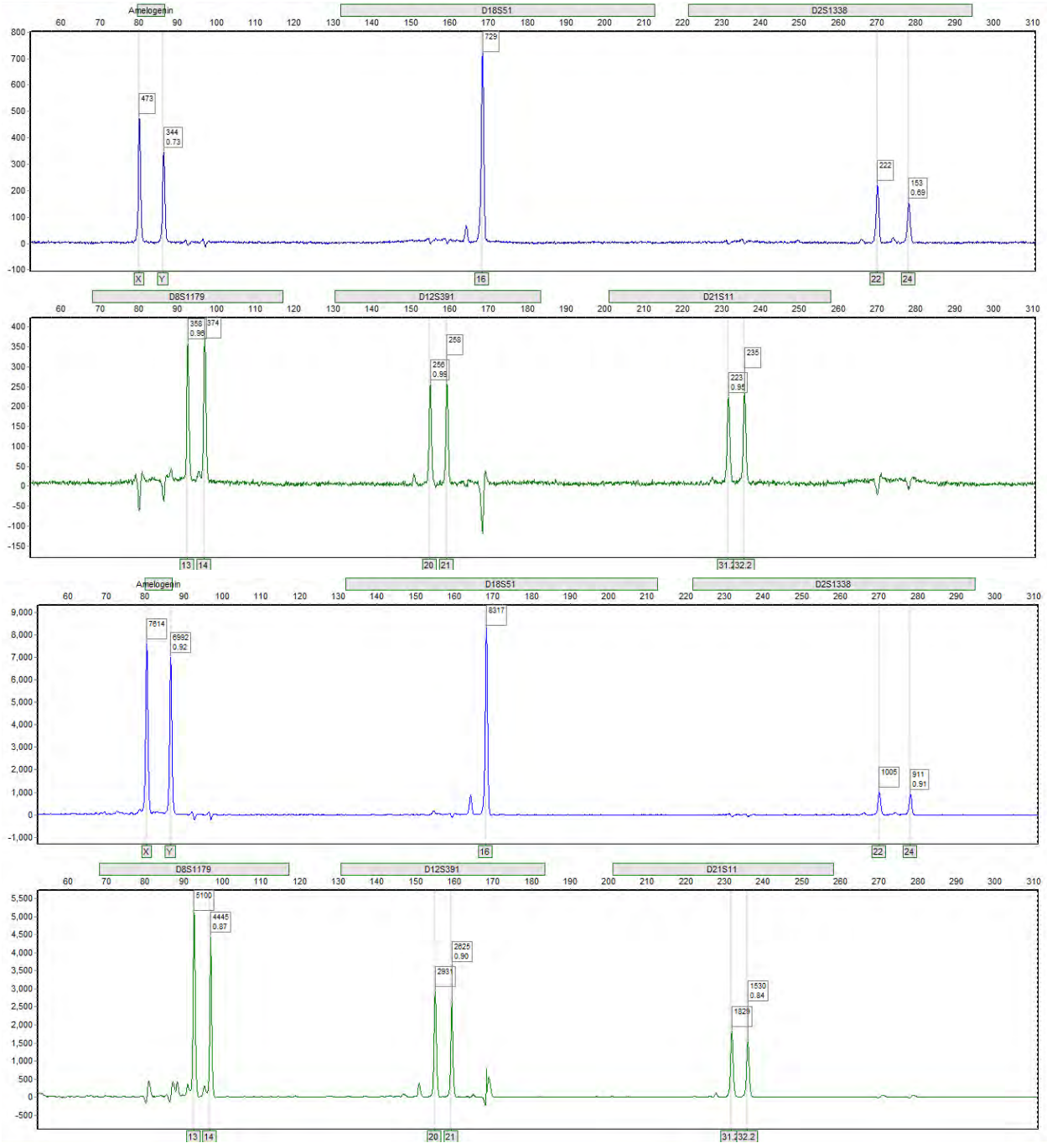


Figure S9: Amplification of the same 20:1 sample with 6-plex and 5-plex PCR. With the removal of the D18 primer, significant improvement in peak heights at all loci is observed.

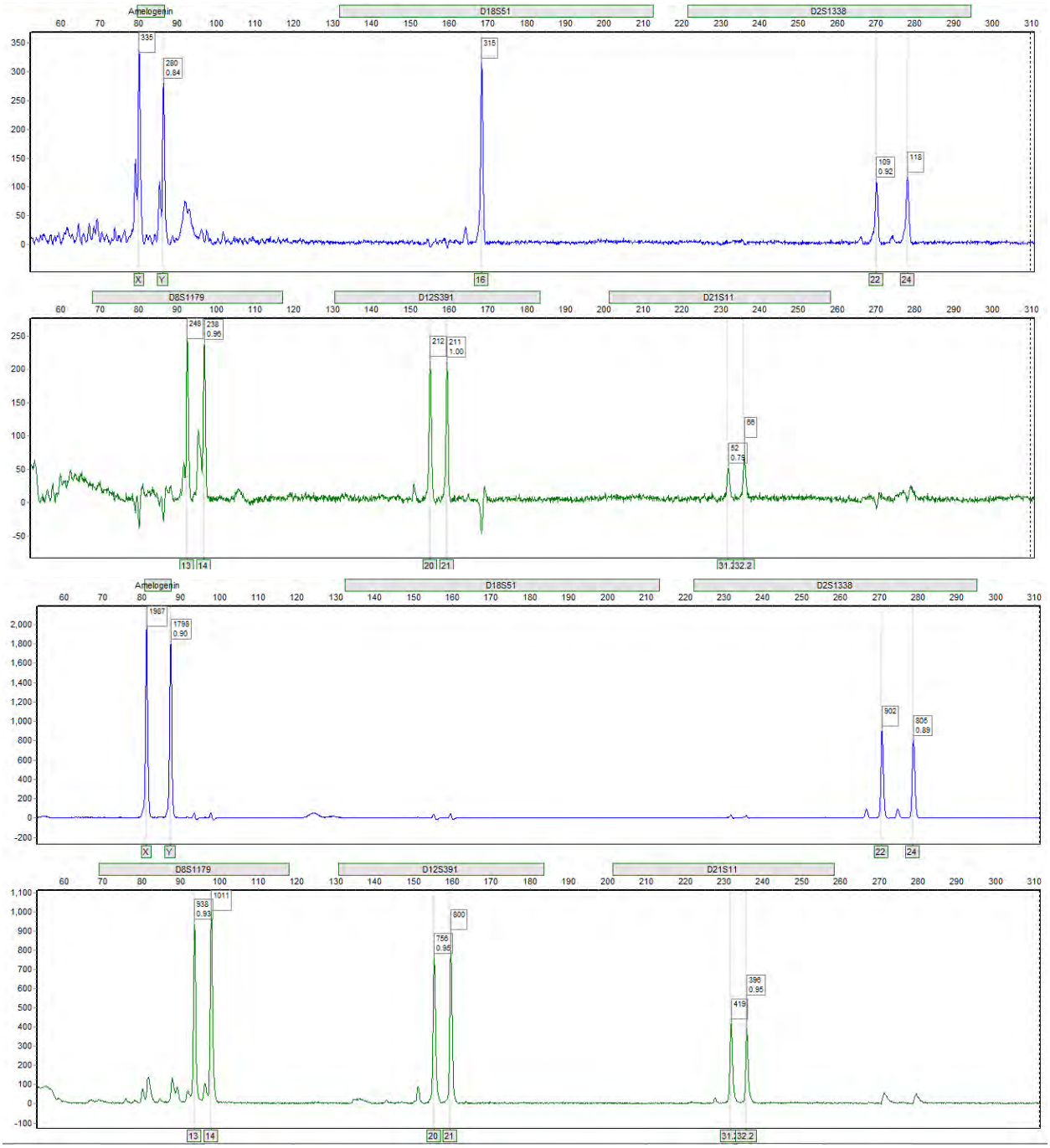
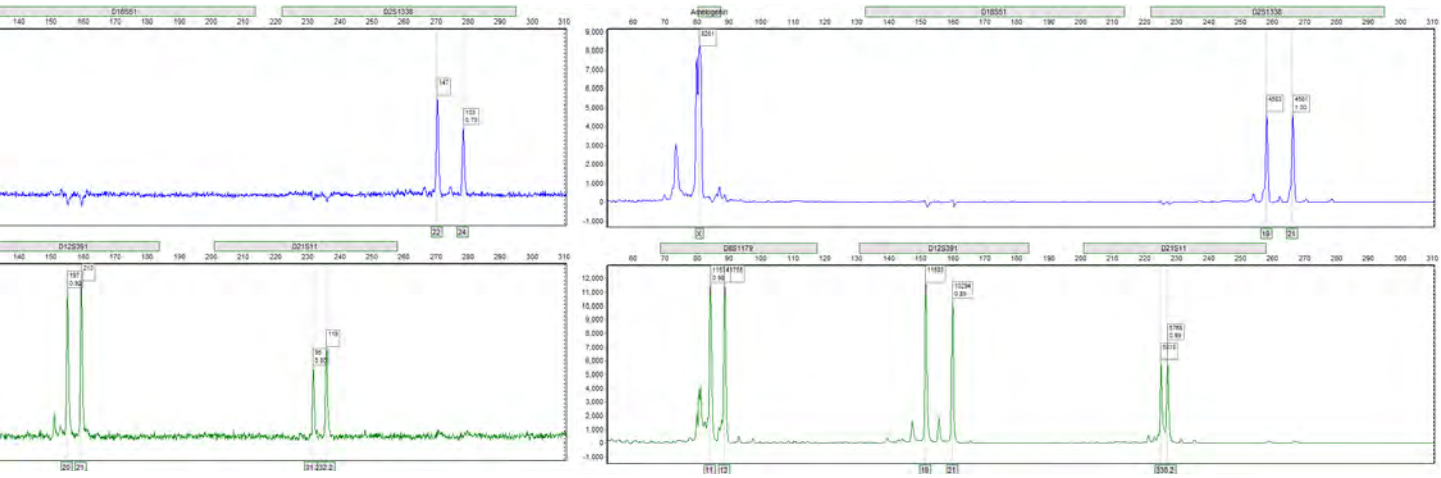


Figure S10: Recovery of failed trapping sample, 30:1 female:male ratio.

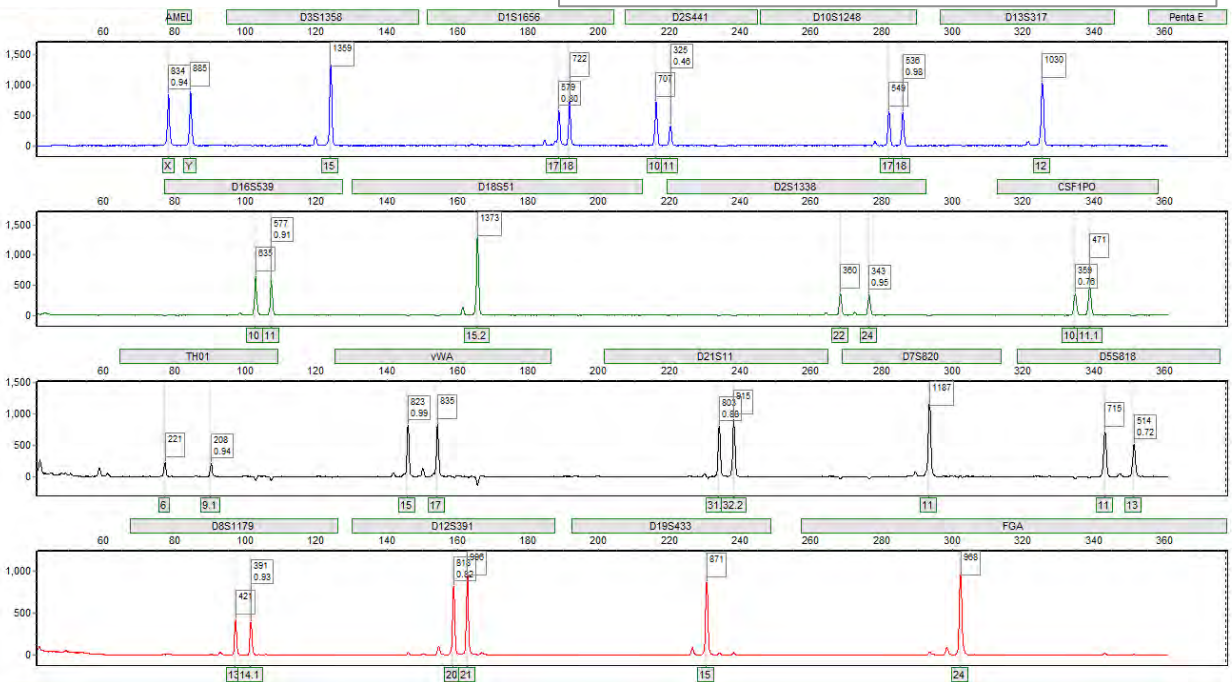


Pre-SONIC profile: only female peaks are present.

Sperm fraction profile: during initial trapping, and aggregate failed to form in the trap site. No sperm pellet was captured.

Re-trapped non-sperm fraction: After initial failed trapping, the non-sperm fraction was re-trapped on a new chip. A sperm pellet was captured, and the resulting profile is fully male.

Neat semen sample from male donor



Male fraction from SONIC run

Figure S11: STR profile for the sample from the male donor for SONIC data given in Figure 4-31. This is the male positive control.

APPENDIX 3 - SONIC DE Standard Operating Procedure

A. Connecting SONIC Instrument and Computer

1. Connect USBs to computer
2. Connect Ethernet to computer to connect Rasberry Pi camera
3. Power on SONIC instrument. You will see lights come on in the front of the instrument.
4. Wait 1 minute before powering on computer
5. Proceed to Part B

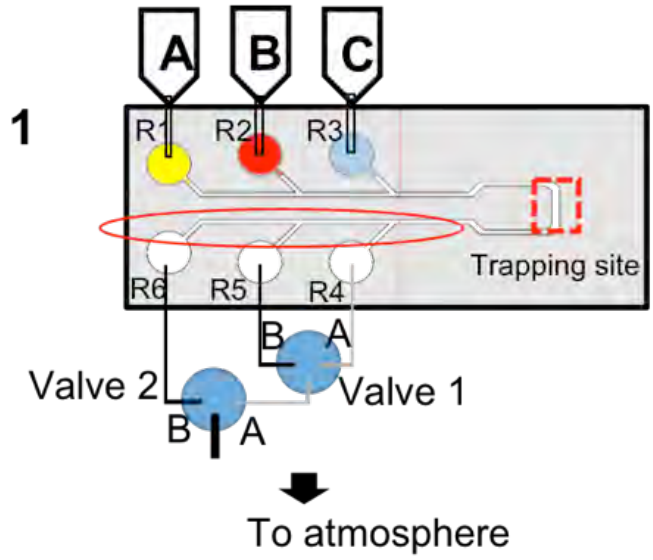
B. Prepare SONIC Instrument

1. Open LabView application
    - Select desktop folder 'UVA Acoustics' → 'Builds' → 'SONIC' folder→Select Application file
    - When application opens, you will be looking at the 'Splash page' on the software
2. Change configuration file
    - Select 'Plots and Configuration' tab
    - Find current configuration file:
        - Select file folder next to 'Configuration file'
        - Computer → C drive → temp → most recent config file (9-a)
3. Click on COM port and choose available port
4. Select "Run" at the top left of the screen (white arrow)
5. Wipe O-rings briefly with a small amount of ethanol on a Kim wipe
6. Step 1: System Initialization
    - Select "Initialize" button in Step 1. This will draw all syringes to the predetermined optimal volumes.
    - A green light followed by a pop of menu will let you know when initialization has completed
7. Proceed to Part C

C. Sample and Device Preparation

1. Prepare sample mixture
    - Vortex pre-lysed sample and sample master mix (assisting beads)
    - In a new polypropylene tube, add 60 µL pre-lysed sample to 10 µL of sample master mix
2. Load sample wells 1-3 (Refer to **Figure 1**). It is critical to load reagents without any bubbles. **Vortex all reagents before adding to the device**
    - R1: Add 10 µL of priming solution directly into inlet within R1. You should see the solution wicking into the device channel. Vortex testing bead solution and then add 110 µL to R1
    - R2: Vortex sample mixture, then add 65 µL to the inlet. Be sure to pop any bubbles that you may introduce

- R3: Add 43 µL deionized H_2O



3. Insert chip into SONIC instrument
    - Seat chip against manifold lip, ensure piezo is aligned over pogo pins
    - Be sure that there are no gaps between the device and the manifold lip
    - **IMPORTANT:** software initialization must be complete before step 3
4. Secure manifold
    - Lower tubing plate onto chip
    - Place trapping plate over trapping site
    - Hold down large manifold plate and close both hinges until they lock into place
5. Close SONIC box top
6. Proceed to Part D

D. Initiating Sample Trapping

1. Press “Start Run” in Step 2 on LabVIEW application
2. Enter optimal trapping frequency that is labeled on the device
3. Click “Continue”. A live feed will start of the trapping chamber. Confirm that trapping occurs with the presence of aggregated particles.
4. A window will pop up asking to choose a snapshot with single largest aggregation of beads. After the selection is made - click “Continue”
5. A live camera feed will pop up and sample trapping will begin
6. Wait ~4 minutes for trapping sequence to finish. Green lights will come on when sequential steps finish. When finished, the software will automatically stop running
7. Select “continue” when the run has completed

E. Retrieving Sample

1. Open SONIC instrument lid
2. Release manifold hinges on manifold with slight finger pressure to prevent abrupt opening of the manifold
3. Remove trapping plate
4. Carefully remove tubing plate without disrupting the microdevice
5. Examine the device to make sure that liquid is in reservoirs 4-6 (**Figure 2**)
6. Take device out of SONIC instrument and extract fractions out of device in the following order using a pipette set to 100 µL: R4 → R5→
7. R6
    - It is important to place pipette in the corner, away from the channel inlet to prevent siphoning liquid
    - Place each fraction in a separate polypropylene tube. The user should expect 30 µL from R4, 60 µL from R5, and 90 µL from R6
8. Proceed to Part B step 6 to rerun another sample
9. When finished with sample running , proceed to Part F

F. Instrument Shutdown

1. At the end of the day
    - Select "Instrument safe shutdown" at the bottom left of the screen
    - If software does not respond, click small "stop sign" symbol at the top left of the screen
2. Close LabView software
3. Shut down laptop
4. Wait 15 seconds and then power off SONIC box

G. Troubleshooting

1. Initialization does not complete
    a. Requires shutting down computer and instrument and rebooting software
2. Snapshots are white screens
    a. Requires shutting down computer while instrument is on and rebooting of software
3. No trapping observed in test bead trapping
    a. Check to see if chip is in correct location
    b. To rerun – simply pipette bead solution back into R1 and try again after re-initializing
4. Video feed fails
    a. Shut down computer and reboot software
5. Videos and snapshot were not saved during run
    a. Computer needs to be shutdown while instrument is on and then software rebooted

ADDENDUM:

Solutions

- Priming solution: EtOH/Glycerol/dI$H_2$0 (5:1:1)
- Test bead solution: 500-fold dilution of bead stock in dI$H_2$0
    - Bead stock: Fluoresbrite YG 6 µm fluorescent particles
- Sample master mix: 650 µL of 6 µm black beads (1/75 black bead stock) + 350 µL of 2 % Tween 20

Chip Cleaning

1. Siphon remaining fluid out of wells using a Kim Wipe
2. Rinse wells with DI H_2O
3. Rinse wells with methanol
    a. Dry the chip using compressed air

Advanced Settings

- If problems trapping, go into advanced options in “configuration tab”
- Place code “2972” into Advanced user box
- Select “Run” (large arrow in top left corner)
- Save a new file
- Change whatever settings necessary

APPENDIX 4 - DISSEMINATION OR RESEARCH FINDINGS

A. MANUSCRIPTS

1. Kerui Xu, Brian L. Poe, Jenny A. Lounsbury, Thomas Laurell and James P. Landers. Bead-Assisted Acoustic Differential Extraction for isolation of a Low Number of Sperm Cells from Female DNA. Submitted *Analytica Chimica Acta*

2. Charles P. Clark, Kerui Xu, Kim Jackson, Anchi Tsuei, Jeff Hickey and James P. Landers. Isolation of sperm cells from simulated sexual assault samples using acoustic differential extraction. In prep *FSI: Genetics*

3. Kerui Xu, Charles P. Clark, Orion Scott and James P. Landers. Microchip design and fabrication for acoustic capture of sperm cells. In prep *J Forensic Science*

4. Orion Scott, Kerui Xu, Charles P. Clark and James P. Landers. Prototyping an instrument for fluidic control in acoustic capture of sexual assault samples. In prep *Rev Scientific InstrumentS*

B. PRESENTATIONS

1. Charles Clark, Kerui Xu, Orion Scott, Kimberly Jackson, Anchi Tsuei, and James P. Landers. Sexual Offender Nodal Isolation of Cells (SONIC): Acoustophoretic separation of sperm cells from mock sexual assault samples. *uTAS-2016 Dublin, Ireland*

1. Charles Clark, Kerui Xu, Orion Scott, Kimberly Jackson, Anchi Tsuei, and James P. Landers. Application of the Sexual Offender Nodal Isolation of Cells (SONIC) System to forensic sexual assault samples. *PittCon-2017 Chicago, IL*

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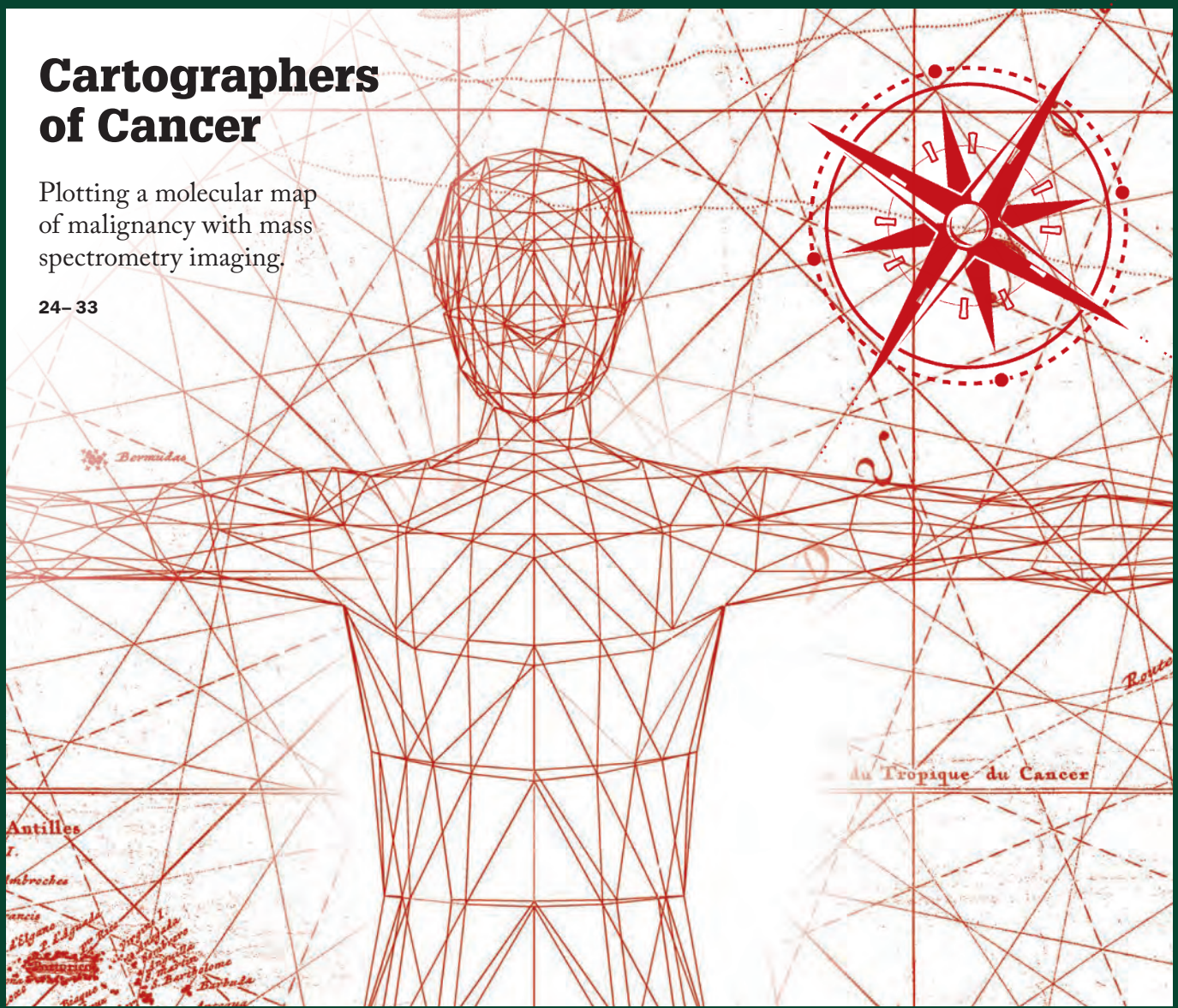
the Analytical Scientist



Cartographers of Cancer

Plotting a molecular map of malignancy with mass spectrometry imaging.

24– 33

speed, simplicity and sensitivity make this method worthwhile for every lab.

The University of Notre Dame does not have a forensics program, so everything I've done has been very reliant on collaboration and communication with other research institutions. The forensics community have been very supportive. We're all passionate about finding ways we can help people – that's what we're in this job for. It's not about fame, making money, or beating your competition – it's about working together to solve society's problems.

I'm very hopeful about the future. There are a lot of people passionate about making progress in forensic science, bringing justice to our communities and lowering crime rates – I want to be part of that.

Sarah Lum is Bioanalytical Chemistry PhD student and Graduate Research Assistant at the University of Notre Dame, Indianapolis, USA.

Battling the Backlog: Part II

With Charlie Clark

I became enamored with acoustic differential extraction (ADE) at graduate school at the University of Virginia. I joined the Landers Research Lab in 2014, and I have since been working on the development of a microfluidic technology (SONIC) that uses acoustic force to separate sperm cells from epithelial cells in sexual assault samples.

Small-scale chemistry

The SONIC system originates from a collaboration that started with Prof Thomas Laurell at Lund Univ and incorporates ADE on a microfluidic device – essentially using sound waves to apply pressure and separate particles. The acoustic trapping principle is the application of a standing sound wave through a microfluidic channel filled with liquid. Those sound waves create low-pressure nodes where they intersect, and high-pressure anti-nodes where the sound waves are out of phase. If you flow particles through that acoustic trapping site, they'll follow the path of least resistance into the low-pressure nodes. And if you tune the frequency of the sound waves properly, you can actually trap and hold particles of a certain size, while everything else flows around it.

Different cell types in the human body vary drastically in terms of size, shape and function. Sperm cells are very well conserved across humans – they're all around 6.0 micrometers in size (at the head) and ~50 micrometers long (head-to-tail), , with roughly the same shape and features. That means we can tune our trapping site very precisely to sperm cells. Once we've flowed our sample through and are holding those sperm cells in place, we have multiple downstream avenues that go to different chambers; we can let all of our sample waste go to one, then switch the flow and release sperm cells into another, thereby purifying those cells that we want to capture.

The conventional method used to separate sperm cells from other cells (primarily epithelial cells) is simple differential extraction. You spin your sample containing multiple cell types at 18,500 x g for 10 or 12 minutes, and the sperm cells will pellet out to the bottom. The analyst removes the supernatant, re-suspends it, and repeats this spin and wash step until they get a purified sperm fraction. It still surprises me that conventional analysis is so manual and thus, how variable this can make the process in handling these types of samples.

In essence, what we're trying to do in the Landers Lab is automate that separation process – taking it out of the hands of the user to make it more uniform. With our methods, you simply load your sample; the metering, fluidic control, trapping, and manipulation are all handled by the instrument – and you are presented with a small vial of purified sperm cells from your sample.

Baby steps

The response to SONIC from the community has generally been positive, although people don't always appreciate the steps that need to be taken in a project like this. When I describe it to other forensic or analytical scientists, they often jump straight to posing convoluted scenarios: "What if you get a sample that has cells from five different people, with four different suspected attackers?" I have to explain that we're not addressing that yet; it takes baby steps to get to that point. What we're doing might not change the types of samples you can look at, but it could open the door to more reproducible male capture – and, in this field in particular, that's crucial.

One of our biggest challenges – and this was unexpected – has been getting reliable information from the rest of the forensic community. We don't have access to real casework. It was really hard, for example, to find out the ratio of female to male cells in a typical sample – we were given numbers that ranged from 1:1 to 600:1.

Probing on Palm Beach

An exciting new development for me was going down to work with Palm Beach County Sheriff's Office (PBSO) in Florida, to observe some of their forensic techniques, train them on using the instrument that we developed, and then compare different extraction methods.

They handed me a list of adjudicated samples – tank tops, sheets, condoms, cheek swabs – all kinds of samples and substrates and cell types that I wasn't ready for. It was much more of a challenge than I thought, but a great opportunity to try the instrument with real samples. One gratifying moment was when they presented us with an adjudicated sample – a cutting from a sheet that had been stored since 2009. We pulled it off the shelf, resuspended it, and were able to separate sperm cells from that sample using the instrument. From our sample, we were able to generate a DNA profile that matched the reference profile that they obtained via their own method eight years earlier. Perhaps not the most challenging sample, but a great moment for us nonetheless.

The trip was really eye-opening. It struck me how unique every lab is; there are different national and state guidelines on how you handle samples, and how you handle these types of investigations. PBSO is a very well-funded state lab, so they have the best instrumentation. It seems like other labs who have obtained less funding may not be able to handle as many samples or hire as many analysts – which means that having new technology that expedites analysis is even more important.

Translating forensics

I'd really had no exposure to forensics before working with this group, but what really hooked me was how easy it is to convey the importance of what I'm working on. Everyone I talk to agrees that it's important to help address the backlog of samples in solving these crimes by speeding up the analysis process. Forensics is in some ways more visible than other areas of analytical science.

Does our technique have scope beyond forensics? We believe so. A recently graduated student from our lab has applied this acoustic isolation technique to the separation of cancer cells. Circulating tumor cells appear in very low numbers in the bloodstream; if you can focus on the differences of those cells – be it in size, shape or compressibility – and separate them using our acoustic technique, then you have the potential to tailor the treatment to the type of cancer the patient has. It's the same principle, but a whole other set of parameters and instrumentation being applied to a new field.

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APPENDIX 5 – Information Request Sent to State Forensic Labs

The information we received back was very non-specific, and each analyst we question responded with some form of "it really just depends on the sample." We were unable to gain any clear understanding of the "typical" sample, but we were led to believe that samples with higher than 50:1 ratios of female:male cells would be quite rare. The questionnaire below was sent to the following labs: CTR U.S. Army, Texas DPS Crime Laboratory, Virginia Department of Forensic Sciences, and OCME New York.

1. How many sperm cells are usually present on an evidence swab?

2. Do you have any data on the quant pre-differential extraction?

3. What is the range of male DNA and female DNA you see in epithelial and sperm cell fractions?

4. What is the approximate final volume of these two fractions in your protocol?

5. Do you know what the typical (or average) amount of sperm and non-sperm DNA is found from sexual assault swabs?

Sample response:

1. There's no amount of sperm that is usually found on an evidence swab. It is highly case dependent. It can range from very few (where we might only find 1 sperm when taking a sample for microscopic exams) to quite a lot (eventually extracting well over 1 ng of DNA).

2. We do not do a DNA quantification pre-differential as that would be pre-extraction. We do microscopic exams, but that is only representative of the small cutting used to make the slide and is not an absolute count of the sperm present from that cutting.

3. I don't have any data on the range, so this is just an estimate. For epithelial fraction we could get tens to hundreds of nanograms of female DNA, male much less. For the sperm fraction I would expect less than 50 ng of male DNA.

4. We extract using EZ1's, so we always elute 50 ul. We have the option to vacufuge concentrate at which point it would be ~12 ul.

5. I couldn't begin to be able to tell you an "average" amount of male/female on an evidence swab (see #1). Whatever the "average" is, the standard deviation would be really large.