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A rotational platform-driven microdevice for differential separation, purification & amplification of sexual assault forensic samples

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Introduction

One of the most common types of cases received in a forensic DNA laboratory are those involving charges of sexual assault/battery, rape, or attempted rape. Often, the biological evidence collected for these cases includes a physical evidence recovery kit, or “rape” kit. These kits are often pre-designed by the lab staff and distributed to first responders, emergency rooms, and sexual assault (forensic) nurses throughout a laboratory’s service jurisdiction, and are used for the standardized collection of DNA from victims and/or suspects involved in a sexual assault case. Types of samples collected in the kits vary and may depend on the victim’s recounting of the assault, but very often include skin and cavity swabs of areas of penetration and ejaculation (ie. body swabs, vaginal swabs, anal swabs, etc.). These types of samples are most likely to include sperm cells (from a male suspect) as well as cells contributed from the victim, from whom the samples are often obtained. The analysis and interpretation of DNA profiles, such as these, that have more than one contributor (ie. “mixtures”) is complex, laborious and leads to high rates of “inconclusive” reporting of results. Fortunately, forensic DNA laboratories have long had the ability to avoid back-end mixture interpretation when sperm cells are present by using front-end differential DNA extraction procedures.

Differential DNA extraction procedures are designed to separate sperm cells from all non-sperm cells (primarily epithelial cells from the victim) prior to cell lysis and DNA extraction. Differential lysis procedures greatly reduce the presence of mixtures (or reduce the presence of secondary contributors) in these samples, allowing for a simplified profile interpretation. Most laboratories continue to rely on manual lysis, microcentrifugation, and manual pipetting steps for physical separation of the two fractions (sperm and nonsperm). This separation process is most often followed by standard manual or automated routine DNA purification procedures (ie. organic or solid-phase DNA extraction methods). Unfortunately, at present, there are few robotic platforms and no known microdevices available that can accommodate an integrated differential lysis process prior to DNA extraction. Until improved platforms are developed and made available to the forensic community that address the specific needs of differential lysis and DNA extraction from sexual assault samples, backlogs will likely continue to grow. Without these, the community will have to continue to rely on manual processes that are lengthy, offer variable results, and require tedious back-end interpretation strategies for mixture deconvolution.

Microdevices offer an alternative to analytical procedures that can be tedious, time consuming, and can lead to highly variable results. However, most researchers interested in microdevice development are focused on μ TAS (micro-total analysis system) – complete integration of laboratory workflow in a single microdevice (ie. “lab-on-a-chip”). While there is impressive work towards development of μ TAS for processing forensic DNA samples, research and development is complex, and full integration, commercialization and implementation of these technologies could take many years. The approach of this research group is shifted more towards making more immediate use of specific microdevice advancements that could replace only a portion of the total workflow, but would improve processing time, minimize variability associated with manual procedures, and shift manual labor to the traditional downstream processes that require examiner time and extensive training (CE analysis, profile interpretation and reporting).

Thus, the approach described herein offers a simple, inexpensive microdevice that utilizes recently developed modules and microfluidic control strategies to replace some (but not all) of the most laborious steps of sample processing for a very common forensic DNA sample type – sexual assault swabs. This approach integrates two existing, recently developed on-chip modules (for DNA purification & IR-mediated PCR) and an existing rotational platform for microfluidic control (1-3) with a novel upstream module for an antibody-based differential separation of sperm and non-sperm cells. With continued efforts, this microdevice could be available for testing, commercialization, and technology transfer within a two year window and could be easily validated and implemented by operational forensic DNA laboratories. Lab

evaluation and implementation would be relatively quick and easy, as this system would replace only upstream analytical wet-lab steps (through amplification), providing a DNA sample and amplified STR products for off-line, unaltered, traditional quantitation and amplicon separation/detection via capillary electrophoresis.

Objective

The overall objective of this project was to design a simple, inexpensive, microchip-based assay that can serve as an alternative to the laborious upstream work that is associated with sexual assault DNA sample processing. This method replaces only the sample prep, sperm/non-sperm cell separation, DNA purification, and multiplex-STR PCR amplification steps of the analytical process, resulting in ready-to-use DNA extract and amplicons that can move straight to an off-chip quantitation and separation/detection via methods that are already validated and standardized in forensic laboratories. This approach minimizes costs, minimizes sample handling, and reduces the labor time that is inherently extended for sexual assault samples that need a differential lysis process.

This project was foundationally based upon a recently developed rotational-device and previously described basic microchip architecture (FSF Lucas Grant 2013, FSF Student Research Grant 2012) from this research group (3). However, several alterations and optimizations had to be completed for specific use with sexual assault samples. First, in order to accommodate the antibody-mediated cell separation on the microchip, the chip architecture had to be redesigned to include an antibody binding chamber and allow for dual valving, microfluidic movement into side-by-side sperm cell and non-sperm cell modules for DNA liberation and multiplex STR amplification. Simultaneously, additional sperm-specific antibodies would need to be screened for their ability to efficiently capture sperm cells to ensure that the very best antibodies (or combination of) could be incorporated into the microchip chemistry. Next, as previous work included only the analysis of buccal swabs, new DNA chemistry would have to be tested to assure efficient lysis of both non-sperm and sperm cells. Lastly, the previously described custom STR multiplex chemistry (2,3) would need reoptimizing using the enhanced buffer STR megaplexes (now commercially available) prior to testing of the integrated microchip system. In the initial proposal, the authors further noted that changes to the system hardware would be tweaked along the way, as needed, to address any microfluidics or valving issues that presented; in the end, hardware upgrades were significant and constituted a major portion of the work completed.

Materials and Methods

Sample Collection & Preparation

Vasectomized and non-vasectomized male volunteers provided semen samples and female volunteers donated vaginal swabs; all volunteers also submitted two buccal swabs for use as reference DNA samples. Buccal and vaginal samples used for flow cytometry and in-tube antibody testing were collected using cotton swabs (Evident™, Union Hall, VA). Any vaginal samples that were collected for processing on a microdevice were collected using foam swabs (Fisherbrand™, Pittsburgh, PA). Only intimate samples with a post coital interval of at least seven days were used in these studies. All vaginal and buccal reference swabs collected for this study were stored at room temperature. Semen samples were divided into 100 µL aliquots and stored at -20°C. All samples used in this study were collected in accordance with VCU approved IRB protocol HMW20002942.

Sexual Assault Microchip Architecture

The design for the sexual assault microdevice was drawn in AutoCAD LT® 2004 software (Autodesk® Inc., San Rafael, CA). The schematics were then exported to VLS 3.5 software to interface with the VersaLaser® 3.50 CO₂ laser ablation system (Universal Laser Systems, Scottsdale, AZ), which

was used to cut the designs from 0.5 mm (top and bottom layers) and 1.0 mm (middle layer) sheets of PMMA (Astra Products, Inc., Baldwin, NY). Layers were bonded using the procedures described in Cox et al. (3).

Evaluation of custom prepGEM® differential method

A custom-modified differential lysis protocol was developed for use with semen/sperm-containing samples using the prepGEM® Saliva kit (ZyGEM™, Hamilton, New Zealand). The performance of this method was evaluated by comparing resulting DNA yields to those obtained using a more traditional differential lysis/DNA extraction method. All semen samples used in this study were diluted 1:2 in PBS solution. For the custom method using ZyGEM™ prepGEM® Saliva for lysis of sperm cells, 10 µL of each diluted semen sample was added to 1 µL of the prepGEM® enzyme, 10 µL of 10X Blue Buffer (ZyGEM™) and 79 µL of water. Samples were heated for 3 minutes at 75°C in a Perkin Elmer GeneAmp 9600 (Perkin Elmer, Waltham, MA) before being spun for 5 minutes at 10,000xg (2). The supernatant was removed and placed into a clean tube labeled non-sperm fraction (NSF). The remaining sperm pellet was then re-suspended in a solution containing 1 µL of the prepGEM® enzyme, 10 µL of 10X blue buffer, 4.5 µL of 1M DTT, and 74.5 µL of water. The tube was heated as described above for 3 minutes at 75°C and was labeled as the sperm fraction (SF).

For samples processed using a traditional differential lysis and DNA extraction approach, 10 µL of each diluted semen sample was lysed by adding 400 µL of stain extraction buffer (10mM Tris, 100mM NaCl, 10mM EDTA, 2% SDS, pH 8.0) and 15 µL of 20 mg/mL proteinase K) followed by a two hour incubation at 56°C. Following incubation, the samples were spun at 10,000xg for five minutes. The supernatant was removed and placed into a new tube labeled non-sperm fraction (NSF). Next, each sample pellet was re-suspended in 200 µL of PBS, 20 µL of Qiagen™ Protease, 20 µL of 1M DTT, 200 µL of Qiagen™ Buffer AL, and incubated for one hour at 56°C. DNA purifications were performed on both fractions (NSF and SF) using the QIAamp® DNA Blood Mini Kit (Qiagen™, Hilden, Germany) according to manufacturer's protocol with a final elution volume of 75 µL.

Antibody evaluation via flow cytometry

An on-going and extensive review of available sperm-specific antibodies was conducted throughout the project period. For a preliminary evaluation of selected antibodies, samples were analyzed by flow cytometry using fluorescently tagged antibodies. To prepare samples for flow cytometry, a modified cell preparation protocol was developed. Vaginal epithelial cell swabs were eluted in 400µl of PBS (Quality Biological, Gaithersburg, MD) at 37°C for two hours with vortexing every 15min. Next, the eluate was divided into two 200µl fractions, one for antibody staining and one for staining with the isotype control. For non-vasectomized seminal fluid, 1:1 dilutions were made with a total volume of 200µl. For vasectomized seminal fluid, 200µl of neat fluid was used for testing. Each seminal sample tested included a separate isotype control tube for staining. For washing, vaginal samples and vasectomized semen samples were first spun down at 400xg for 5mins while semen samples were spun at 800xg for 5 mins; following these spins, the supernatant was discarded. The cells were then resuspended in 300µl of cell staining buffer (Southern Biotech, Birmingham, AL). This wash/spin process was repeated an additional two times.

Next, 25µl of a 0.16mg/ml solution of rabbit IgG (GenScript, Piscataway, NJ) was added to each sample tested along with 5µl of FcR Blocker (Miltenyi Biotec Inc., San Diego, CA); the blocker was included in order to block any non-specific binding sites expressed on the cell membrane. The samples were covered and incubated for 10-20 mins at 4°C. Then, 25µl of a 20ng/µl solution of the appropriate antibody or isotype control was added to each sample; samples were again covered and incubated for an additional 35 mins at 4°C. After this staining process, the samples were spun down at 400xg for 5 mins. The supernatant was removed, and the cells were resuspended in 300µl of cell staining buffer. This process was repeated an additional two times. After the third wash, cells were resuspended in 200µl of cell staining buffer. Samples containing vaginal epithelial cells were pipetted through a nylon mesh strainer prior to

flow cytometry to prevent cell clumping.

All stained samples were processed on a FACSCelesta™ flow cytometer (BD Biosciences, San Jose, CA). Samples were gated based on cell size and granularity to best capture the stained cells of the targeted cell population. Data was recorded until either 100,000 events were detected, or until after 45 seconds passed. SP-10 (Bioss), PH20 (LSBio), hK2 (CUSA Bio), and PSA (CUSA Bio) were all tested in triplicate, and AKAP3 (Aviva Systems Biology) was tested in duplicate. For data analysis, a threshold was set with the IgG isotype control against which each sister sample was compared. Samples were gated based on the size and granularity of the cell of interest for this comparison of the control with the sister sample. The cell count value obtained for the isotype control fluorescence was subtracted from the value obtained for the sample stained with the tested antibody to generate a percent of cells within the gated population that had stained positive for the selected antibody. Replicate values obtained for each sample tested were averaged. An analysis of variance (ANOVA) statistical test ($\alpha=0.05$) was used to compare the percent of positive cells noted for each antibody among the cell populations tested. This analysis was coupled with a Tukey HSD post-hoc analysis when significant differences were noted.

Bead-mediated sperm cell capture

Streptavidin-labeled polystyrene beads ~200 microns in size and 1% w/v (Spherotech, Lake Forest, IL) were selected for use in this study. In order to bind the tested antibodies to the beads, 9 μ L of bead solution (approximately 20 beads) per sample to be processed was added to a total of 1 mL of PBS followed by a spin at 100xg for 60 s. The supernatant was discarded and the wash step was repeated twice more. After the final spin, the supernatant was discarded and ~0.167 μ g of the biotin-labeled antibody was added for each sample to be processed. The bead:antibody mixture was incubated at room temperature for 30 minutes with gentle agitation. Following incubation, five additional washes were performed with 1 mL PBS as described above. After the final spin, the bead pellet (containing the antibody-coated beads) was resuspended in 9 μ L of PBS per sample to be processed. In order to bind target cells to the antibody-coated beads, 9 μ L of the antibody-coated bead solution was added to 10 μ L of a 1:2 semen dilution for each sample tested. The samples were incubated for 35 minutes at room temperature (to bind the sperm cells) and then spun at 100xg for 60 s.

The supernatant was removed into a new tube for DNA preparation. For DNA preparation of bound cells, the bead pellet was resuspended in 1 μ L of the prepGEM® enzyme, 10 μ L of 10X Blue Buffer (ZyGEM™), 4.5 μ L of 1M DTT and water to 100 μ L total volume. The supernatant fraction prepped by adding 1 μ L of the prepGEM® enzyme, 10 μ L of 10X Blue Buffer (ZyGEM™), and water to 100 μ L total volume. Both fractions (supernatant and bead pellet) were then heated at 75°C for three minutes prior to DNA quantification as described above. To provide a basis for comparison, two additional sample groups were processed – samples incubated with no antibody-coated beads (“no beads”) and samples incubated with non-coated beads (“naked beads”). All samples were tested in duplicate.

On-chip bead-mediated sperm cell capture (initial device)

After successful bead-mediated antibody binding results were observed using the simple chip design, the bead capture and liquid separation mechanisms were tested using the proposed integrated sexual assault microdevice (**Figure 1**). Antibody-coated beads for testing on the sexual assault microdevice were prepared prior to testing, as described above, except the antibody-coated beads were ultimately resuspended in an 8 μ L mix containing 0.8 μ L 10X Blue buffer (ZyGEM™), 0.32 μ L prepGEM® enzyme (ZyGEM™), and 6.88 μ L water.

For microdevice runs, one sixth of a dry semen, vaginal, or mock postcoital swab ($n = 3$ for each) was placed in the swab chamber with 1.8 μ L 10X Blue buffer, 0.72 μ L prepGEM® enzyme, and 15.48 μ L water, and the swab was agitated with a pipet tip to loosen cells. The swab cutting was then removed and 8 μ L antibody-coated bead mixture (described above) was added. The swab chamber was sealed with PCR film to prevent evaporation, and the sample was incubated in the microdevice at room temperature for 35

min. Following incubation, all mechanical valves were closed, and the microdevice was spun 2 min at 500 rpm using the custom built rotational device (described above) to move the unbound cells through the burst valve into the unbound ZyGEM™ chamber. Next, a mix containing 1.4 µL 10X Blue buffer, 0.56 µL prepGEM® enzyme, 0.63 µL 1M DTT, and 11.41 µL water was added to the bound ZyGEM™ chamber. The ZyGEM™ reference chamber was filled with water and a type T thermocouple (Physitemp Instruments, Clifton, NJ) was inserted, all ports on the chip were sealed with PCR film, all mechanical valves were closed, and the chip was heated for 3 min. at 75°C on a custom-built IR-PCR device (3-5). After heating, mechanical valve #1 was opened, and the liquid in the bound ZyGEM™ chamber was spun through into the holding chamber for 2 min at 800 rpm. Both bound and unbound fractions were then manually removed from the microdevice for downstream processing.

For comparison, another one sixth of each postcoital swab tested on the sexual assault microdevice (n = 3 for each) was processed using each of the standard tube-based differential lysis and DNA purification methods described above (custom modified PrepGEM® method and QIAamp® method). Finally, one sixth of each postcoital swab was processed without a differential lysis procedure at all with DNA extraction using the QIAamp® DNA Blood Mini Kit (Qiagen™, Hilden, Germany) according to manufacturer's protocol with a final elution volume of 75 µL. DNA quantification was achieved for all tested samples using the Investigator® Quantiplex HYres kit (Qiagen) method, as described below.

Microdevice troubleshooting & modification (v2)

In order to address limitations of the v1 microdevice and spin/heating platforms, significant changes were made to both the microdevice materials (consumable) and the system hardware. The new microdevices are made from polyester/toner, rather than PMMA, and are cheaper, faster to fabricate, and allow for faster, easier, and more automatable valves. The new hardware allows for faster/easier mounting of the microdevice, faster acceleration and deceleration, heating and spinning on the same platform, and is connected to a user-friendly laptop interface that allows for much finer spin control. This newly redesigned system was used to assess the ability of the device to separate sperm from non-sperm cells using the bead-mediated method described above and SPAM1/PH-20 (sperm-specific antibody). Three mock post-coital samples were tested using the v2 system and forensicGEM™ Sperm liberation chemistry (ZyGEM). Bound and unbound DNA samples were amplified off-chip using the AmpFℓSTR™ Identifiler™ PCR Kit (Life Technologies) STR amplification conditions described below.

DNA quantification

The Investigator Quantiplex® HYres kit (Qiagen) was used to quantify the DNA present in each sample. For this, a 7500 Real Time PCR System (Life Technologies, Carlsbad, CA) was utilized; protocols for quantification followed the manufacturer's protocol with the exception of using half volume reactions. To analyze any data obtained from quantification, the total DNA yield was first calculated for all samples by multiplying the concentration (ng/µl) of each sample by the total volume (100µl). The percent total DNA in the bound and unbound fractions were calculated and compared for all single source samples by dividing the bound or unbound fraction yield by the sum of the total DNA yield for the bound and unbound fractions. These values were compared using an ANOVA ($\alpha=0.05$) coupled with Tukey HSD *post hoc* analysis when appropriate ($p < 0.05$).

Amplification and separation through capillary electrophoresis

The AmpFℓSTR™ Identifiler™ PCR Kit was used for STR amplification of all samples evaluated in the experiments described above. Modified reaction volumes were used consisting of 5.7µl AmpFℓSTR™ PCR reaction mix, 2µl AmpFℓSTR™ Identifiler™ Primer Set, 2.1µl TE⁻⁴, 0.2µl (one unit) AmpliTaq Gold DNA polymerase, and 5µl of sample (containing 1ng template DNA input). Samples were amplified on a GeneAmp 9600 (Perkin-Elmer) with the following parameters: pre-denaturation 11 min. at 95°C, followed by 28 cycles of: 94°C, 59°C, and 72°C for 60s each, and a final extension step at 60°C for 90 min. Following amplification, samples were separated on a 3130 genetic analyzer (Life Technologies)

Amplified sample (1.5 μ l) or 1 μ l of allelic ladder was added to 12.0 μ l of Hi-Di formamide (Life Technologies) and 0.1 μ l of GS 500-LIZ (Life Technologies) CE parameters for this run included 3kV 10s injections using a 36cm capillary and POP-4 polymer (Thermo Fisher Scientific, Waltham, MA).

In a final experiment, swabs from three individuals were used to test the custom on-chip STR amplification chemistry described previously (3), but with primers from PowerPlex[®] Fusion 5C (Promega, Madison, WI), a more recently released multiplex STR amplification kit. One twelfth of a buccal swab from each individual was liberated on-chip for 5 min. at 75°C, whose reaction consisted of 16.72 μ l ddH₂O, 1.9 μ l 10X Blue Buffer (ZyGEM) and 0.38 μ l forensicGEM[™] enzyme (ZyGEM). For comparison, a single buccal swab from each individual was also extracted using the QIAamp Blood Mini Kit (Qiagen) per manufacturer's recommendations. All samples were amplified on-chip using the v2 system (described above) and the reaction and thermalcycling conditions previously described (3), but with 2.5 μ l of PowerPlex[®] Fusion 5C primers rather than Identifiler[™] primers.

STR data analysis

STR data from the 3130 Genetic Analyzer was analyzed using GeneMapper ID v4.0 (Life Technologies) using an analytical threshold of 75rfu. The allele calls for each sample were compared against the reference profiles, and the number of alleles observed was compared to the expected number of alleles. Major:minor peak height ratios ("male:female ratios") were calculated by dividing the sum of the peak heights of the minor contributor by the sum of all four peak heights. Data from all sample groups was compared using ANOVA statistical test ($\alpha=0.05$) followed by *post hoc* Tukey tests when appropriate ($p < 0.05$).

Results and Discussion

Sexual Assault Microchip Architecture

The schematic showing the proposed architecture for the sexual assault microdevice is shown in **Figure 1**. This design takes advantage of the small width of the burst valve channel to separate the sperm and non-sperm cells. With the sperm cells bound to the ~200-micron antibody-coated beads, they would no longer be able to pass through the burst valve (~100 microns) when the microdevice is spun using the previously developed rotational platform (3). Thus, the first spin, (designed to open the burst valve) would allow only unbound (non-sperm cells) to move down the right side of the microdevice to the unbound ZyGEM[™] DNA preparation chamber, leaving the sperm-bound bead complex in the antibody binding chamber. Next, a ZyGEM[™] mix containing DTT is added to the bound ZyGEM[™] chamber and the entire microdevice is moved to the IR-PCR device for simultaneous DNA liberation. After heating, the first mechanical valve can be opened, allowing the liberated DNA from the bound ZyGEM[™] chamber to be spun through to the holding chamber. All architecture downstream of the DNA preparation chambers is identical to that developed and tested with the initial swab microdevice described by Cox et al., but allowing for side-by-side processing of the bound (sperm) and unbound (non-sperm) fractions (3). IR-mediated STR amplification was previously optimized and described for use with this type of microdevice (3). Thus, after separation of sperm and non-sperm cells in the upstream modules and a brief ~45 minute amplification step, the PCR products can be easily removed from the sexual assault microdevice for traditional CE-based fragment separation and detection.

Evaluation of custom prepGEM[®] differential method

The proposed sexual assault microdevice architecture described above relies on the use of an enzyme-mediated DNA liberation assay (previously described). However, there are no reports of this method for use with sperm cells, which require special lysing conditions to disrupt the rigid acrosomal cap that protects the sperm cell head. Thus, a custom method was developed which uses a modified ZyGEM[™] prepGEM[®] Saliva protocol. Samples processed with the modified ZyGEM[™] prepGEM[®] Saliva method

had an average sperm fraction yield of 64.99 ± 17.98 ng compared to QIAamp® DNA Blood Mini Kit at 15.75 ± 14.48 ng (**Figure 2**, $p=0.093$). When comparing non-sperm fractions, samples processed with the modified ZyGEM™ prepGEM® Saliva method had an average yield of 178.1 ± 172.6 ng whereas QIAamp® DNA Blood Mini Kit only contained only 73.85 ± 28.47 ng on average ($p=0.409$). These results show that the experimental modified differential ZyGEM™ prepGEM® can perform comparably to traditional differential lysis methods and is suitable for use with the proposed sexual assault microdevice.

Antibody selection

Following a review of relevant literature, antibodies that could target a the sperm cell contributor from sexual assault mixture samples were identified (**Table 1**). Each candidate antibody was ranked based on the expression level on the sperm cell membrane, species specificity, and target (sperm) cell specificity. Alternately, CK4 was also selected as a candidate to be tested based on its known ability to specifically select for binding (female) vaginal epithelial cells (6-8).

Antibody testing by flow cytometry

Using our optimized flow cytometry protocol, SPAM1/PH- 20 showed a higher binding efficiency for sperm cells than all other sperm antibodies tested (**Table 2**), binding 74.18% of positively stained sperm cells (**Figure 3**). Further, SPAM1/PH-20 and SPAG-8 showed a higher binding affinity for sperm cells when compared to AKAP3, which bound a minimal number of cells.

Bead-mediated sperm cell capture

Several antibodies were selected for additional testing (in-tube) using the antibody-coated, bead-mediated mechanism proposed. SPAM1/PH-20, AKAP3, and MOSPD3 were least successful in sperm capture using this approach, as less than 50% of the total sample DNA was obtained from the bound fraction when semen samples were tested (**Table 3**). SPAG-8 (70.09%) and CRISP2 (52.61%) outperformed all other antibodies tested, although both provided a slightly lower percentage of DNA than expected based on known seminal cell populations. Cytokeratin 4 was also tested as it has been shown to successfully bind mucosal epithelial cells, the non-sperm cell portion of seminal fluid. When tested on semen samples, it performed with similar efficiency to SPAG-8, leaving 68.98% of DNA in the *unbound* fraction (**Table 3**).

Mixtures of semen:vaginal samples were also tested using the tube-based antibody-coated bead assay described above in order to determine which could best preferentially target and identify the male contributor. Interestingly, the STR profiling results showed that SPAM1/PH-20, AKAP3, and CK4 outperformed SPAG-8 and CRISP2 (**Table 4**). In these mixtures, the male contributed 10-fold more to the resulting STR profiles from the bound fraction (SPAM1/PH-20, AKAP3) or unbound fraction (CK4) than did the female and often resulted in single-source male profiles (**Figure 4**). This is ideal, given that many laboratories report that mixtures containing a major profile present at $\geq 10:1$ major: minor ratios are often interpretable as extrapolated single-source profiles given the very low to undetectable presence of the minor (9). Unfortunately, the male was also the major contributor to the STR profiles obtained from the alternate fraction from these samples as well (**Table 4**). This indicates that a percentage of the male (sperm) cells are not captured by the bead-mediated mechanism when sperm-specific antibodies are used and are therefore leaking into the unbound fraction. While this is not ideal, obtaining a clean, easy-to-interpret male profile in the bound fraction is the primary goal of mixture cell separation – and this was achieved. Future work will seek to optimize the binding conditions of this assay to assure that all target cells are bound and remain in the bound fraction.

On-chip bead-mediated sperm cell capture

For this test, SPAG-8 antibody-coated polystyrene beads were incubated with seminal fluid directly in the microdevice. Use of the SPAG-8 antibody-coated beads resulted in a 39% increase in the amount of sperm cell DNA captured in the microchip environment when compared to incubation of the same samples without antibody-coated beads (**Figure 5**). When compared to results obtained off-chip using a traditional

differential lysis and DNA extraction methods, a significant increase in the amount of sperm cell DNA captured was also observed (**Figure 5**, $p=0.080$). When semen and vaginal samples were tested on the integrated microdevice, expected male STR profiles were obtained in the bound fraction of the semen samples and expected female STR profiles were obtained in the unbound fraction of the vaginal swab samples, as desired (**Figure 6**).

Microdevice troubleshooting & modifications

Initial testing of the proposed microdevice (**Figure 1**) revealed several significant limitations. First, the mechanical valves used in the proposed architecture caused the beads (used for cell capture) to clog, which led to delays in the fluidic movement between chambers. Further, mechanical valves require manual (or pump actuated) manipulation to open and close, which would ultimately require a more complex hardware system with a larger footprint. With this in mind, alternative valve methods and microdevice materials were explored. Laser/tap valves replaced mechanical valves in the overhauled microdevice platform design (“v2”). This valving technology employs a laser to physically create a hole in the plastic material through which liquid can easily flow through, once opened. This approach is much faster than the manual opening of mechanical valves and is much more amenable to automation for future prototypes. Additionally, the microchips are now constructed from layers of polyester transparency sheets, heat sensitive adhesive, and printer toner (**Figure 7**). The five-layer design is slightly less expensive to produce and requires a similar amount of time for laser ablation, but the bonding process is over an order of magnitude faster. Further, these new materials provide the ability to employ the new laser/tap valving system for microfluidic control.

In order to accommodate changes made to the microdevice itself, the accompanying hardware system required significant upgrading. The new spin motor in v2 allows finer control over spin speeds and lengths, as well as considerably faster acceleration and deceleration. These parameters are controlled through a laptop user interface, which is a quantum leap forward when compared to the manual dial on the voltage regulator used in the previous system. The updated software for the newly modified platform includes a shaking/mixing function that may be beneficial in assisting the bead-cell interaction. In addition to the spin motor and software, the new hardware system also houses the heating system used for DNA liberation and PCR. The new heating system employs a Peltier clamp instead of the IR-PCR initially used, providing direct heating and cooling with similar ramp times.

Initial fluidic testing of the modified v2 sexual assault microdevice platform has successfully mimicked the workflow followed by the previous microdevice, however, in v2, the microdevice required manual removal from the mount and rotation in order to alter the direction of fluidic movement. Since this process does not lend itself well to future automation, additional modifications were made to the spin platform (“v3”). The current hardware (v3) includes the addition of servo motors on either side of the center of rotation (**Figure 8**), which allow for an automated rotation of the microdevice. Further, by changing the orientation of the device relative to the center of rotation, beads and cells can be moved to different locations within a chamber, which increases the likelihood of the beads coming into contact with their target cells and improves mixing capabilities (**Figure 9**).

Initial testing of the newly redesigned microdevice and system hardware (v3) has recently commenced. Cell-separation capabilities were tested using mock postcoital samples and a sperm specific antibody, SPAM1/PH-20. Unfortunately, in this preliminary test, both the bound and unbound fractions showed clear mixtures at most loci (**Figure 10**). However, the bound fraction showed a male:female peak height ratio of 2.96:1, while the unbound fraction showed a ratio of 1.22:1, demonstrating 142% enrichment for the male profile in the bound fraction. Qualitatively, fluid movement and user friendliness on this new platform are significantly improved, and with further optimization of the architecture and cell capture chemistry (pending new funding), the mixture separation will undoubtedly improve.

PowerPlex® Fusion 5C custom STR chemistry

Over the course of this project, the Identifiler™ STR amplification chemistry has become obsolete

and the community has shifted to newer, larger multiplex assays that have enhanced buffering chemistries. Thus, we have begun evaluating the use of the PowerPlex[®] Fusion 5C primers in the custom on-chip STR amplification previously described (3). In-tube amplification of conventional single-source Qiagen-extracted DNA using our custom STR reaction and thermalcycling conditions, but with PowerPlex[®] Fusion 5C primers (rather than Identifiler[™]), produced full STR profiles with strong balance and average peak heights of ~2800 rfu. When forensicGEM -liberated DNA was similarly amplified, strong partial STR profiles were produced (83% of expected alleles), however, weaker inter-locus balance was observed and average peak heights reduced to ~800 rfu (**Figure 11**). Although optimization is needed (pending additional funding), this data suggest that PowerPlex[®] Fusion 5C primers can work as well as those previously used; consequently, PowerPlex[®] Fusion 5C primers will be used in all future work on this project.

Conclusions & Future Implications

The goals of this project were to develop and test a microfluidic-based microdevice, along with associated chemistry and hardware that could be used for automated front-end processing of sexual assault forensic samples. We have described herein a novel antibody-based bead-mediated assay for cell capture and “differential” isolation of male sperm cells as well as a cell lysis protocol that improves recovery of sperm DNA. The microdevice architecture that has been developed is capable of cell-separation with downstream, side-by-side DNA liberation and includes a custom rapid multiplex STR PCR to provide CE-ready amplicons in under 90 minutes. With additional optimization, validation and subsequent commercialization, the sexual assault microdevice developed and described herein could have an immense impact on the forensic science community. First, it could significantly reduce the amount of hands-on processing time spent on processing of sexual assault evidence samples. This would allow for a shift of labor to more complex, tedious processes of DNA profile review, interpretation, and reporting. Secondly, the utilization of sperm specific antibodies for physical separation of sperm and non-sperm cells improves upon current manual techniques, the success of which is varied and based largely on the pipetting skill and experience of the individual technician performing the differential separation. The automation and standardization of this cell separation process should further lend itself to a reduction in the large number of mixtures typically observed (and thus, examiner interpretation time) with sexual assault samples when manual, traditional procedures are employed. While currently available “Rapid” μ TAS systems would ultimately solve many of these issues, it may take years to get there for forensic sample processing. In the meantime, providing labs with simple, inexpensive, automated tools for completing the most labor-intensive laboratory steps of the forensic DNA workflow allow them to take advantage of microdevice modular technologies that are already well optimized. Further, using these systems to improve front-end processing steps avoids the need for time-consuming validations associated with the implementation of μ TAS and/or new electrophoretic platforms and software that more directly impact data quality and profile interpretation.

Although this work has made significant strides towards development of the proposed sexual assault microdevice system, there are several areas for improvement that would be beneficial. While our work has identified sperm-specific antibodies that are very efficient at binding the male contributor in mixture samples, non-specific desorption (cell leakage) affects the unbound fraction quite significantly, as the major profile in the unbound fractions is often the male contributor. There are several hypotheses that could explain the inefficient binding that should be explored with future work, including non-specific binding of non-target cells, non-specific desorption, and sub-optimal antibody binding temperature. Additionally, modifications to the binding mechanism and/or antibody delivery methods could further improve binding and should be explored. It is possible that the size, material, or traits of the beads, the antibody density, the STR amplification kit being used, the antibody orientation with regards to the

streptavidin-coated bead, and the position of the biotinylated tag on the antibody are also factors affecting target cell binding. Lastly, alternative ligands should be evaluated as potential mechanisms for cell capture, such as sperm-specific aptamers.

Ultimately, the developed hardware and sexual assault microdevice offer a simple, robust approach that is easy to use, which should reduce the training time associated with differential lysis and DNA extraction. These time savings, coupled with the low cost of the materials, reagents, and associated hardware would be expected to result in significant overall cost savings to a forensic DNA laboratory. Further cost savings could be achieved with a small increase in the size of the microdevice to accommodate multi-sample processing. Lastly, commercialized prototypes of this system offer an inexpensive solution with a small footprint, which improves the potential for portability.

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Products

Presentations:

1. American Academy of Forensic Sciences 69th Annual Meeting (2017), New Orleans, LA. A Novel, Extraction-PCR Microdevice on a Rotation-Driven Platform: Buccal Swab to STR

- Product in Less Than Two Hours. Jordan O. Cox, MS, Teresa K. Sikes, MS, Kemper Gibson, MS, Cathey Connon, PhD, Kimberly R. Jackson, PhD, James Landers, PhD, Tracey Dawson Cruz, PhD.
2. American Academy of Forensic Sciences 69th Annual Meeting (2017), New Orleans, LA. Microchip-based Antibody-Mediated Differential Lysis of Sperm Cells. Molly E. Woodson, MS; Kemper Gibson, MS; Jordan Cox, MS; Kimberly Jackson, PhD; James P. Landers, PhD; Tracey Dawson Cruz, PhD.
 3. VCU Forensic Science Department Seminar Series: Spring 2017, DNA, Microchips, and More: Moving beyond the mystery of the big magic box Tracey Dawson Cruz, PhD. Richmond (VA): Virginia Commonwealth Univ. 2017.
 4. A Microchip Module for Antibody-Mediated Differential Separation of Non-Seminal Male/Female Mixtures from Sexual Assault Samples [thesis]. Molly E. Woodson, MS. Richmond (VA): Virginia Commonwealth Univ. 2017.
 5. Mid-Atlantic Association of Forensic Scientists 2017 Annual Meeting, Pittsburgh, PA. A Microchip Module for Antibody-Mediated Differential Separation of Non-Seminal Male/Female Mixtures from Sexual Assault Samples. Molly E. Woodson, MS; Kemper Gibson, MS; Jordan Cox, MS; Kimberly Jackson, PhD; James P. Landers, PhD; Tracey Dawson Cruz, PhD
 6. Mid-Atlantic Association of Forensic Scientists 2018 Annual Meeting, Hunt Valley, MD. Forensic sample processing using an updated rotational platform, Peltier heating, and polyester-toner microdevices. Jordan O. Cox, MS; Killian O'Connell, BS; Shane Woolf, MS; James P. Landers, PhD; Tracey Dawson Cruz, PhD.
 7. Mid-Atlantic Association of Forensic Scientists 2018 Annual Meeting, Hunt Valley, MD. Antibody mediated separation of seminal male/female mixtures from sexual assault samples. Chelsie Testerman, MS; Jordan Cox, MS; James P. Landers, PhD; Tracey Dawson Cruz, PhD.
 8. A Microdevice Module for Antibody Mediated Separation of Seminal Male/Female Mixtures from Sexual Assault Samples [thesis]. Chelsie Testerman, MS. Richmond (VA): Virginia Commonwealth Univ. 2018.
 9. Gordon Research Conference: Forensic Analysis of Human DNA (2018), Newry, ME. Antibody selection and preliminary testing for bead-mediated cell separation: Preparing for integration on an updated centrifugal sexual assault microdevice platform. Jordan O. Cox, MS; Chelsie Testerman, MS; Killian O'Connell, BS; James P. Landers, PhD; Tracey Dawson Cruz, PhD.
 10. American Academy of Forensic Sciences 71st Annual Meeting (2019), Baltimore, MD. Antibody-Mediated Separation of Seminal Male/Female Mixtures from Sexual Assault Samples. Chelsie Testerman, MS; Jordan O. Cox, MS, Kemper Gibson, MS, Landers, PhD, Tracey Dawson Cruz, PhD.
 11. Mid-Atlantic Association of Forensic Scientists 2019 Annual Meeting, Morgantown, WV. Evaluation Of Sperm-Specific Antibodies For Bead-Mediated Cellular Separation Of Sexual Assault Samples. Yolanda Correia, MS, Jordan O. Cox, MS; Brittany C. Hudson, MS, James P. Landers, PhD; Tracey Dawson Cruz, PhD.
 12. International Symposium on Human Identification 2019, Palm Springs, CA. A Novel Differential Protocol for Sexual Assault Samples using a Target-Specific Antibody-Bead Complex. Chelsie Testerman, Jordan O. Cox, Molly Woodson, Yolanda Correia, Brittany Hudson, Kemper Gibson, James P. Landers, Tracey Dawson Cruz.

Technologies or techniques:

1. Patent filed CRU-16-033 (Disclosure Accepted May 2016; Full Application filed March, 2017) Title: A rotational platform-driven microdevice for differential separation, purification, and amplification of sexual assault forensic samples

Other products:

1. Cox JO, Sikes T, Connon C, Jackson K, Ouyang Y, Strachan B, Nelson D, Thompson B, Sloane H, Landers JP, Dawson Cruz T. (2016) A novel, integrated forensic microdevice on a rotation-driven platform: buccal swab to PCR product in less than two hours. *Electrophoresis* 37(23-24):3046-3058.

Tables & Figures:

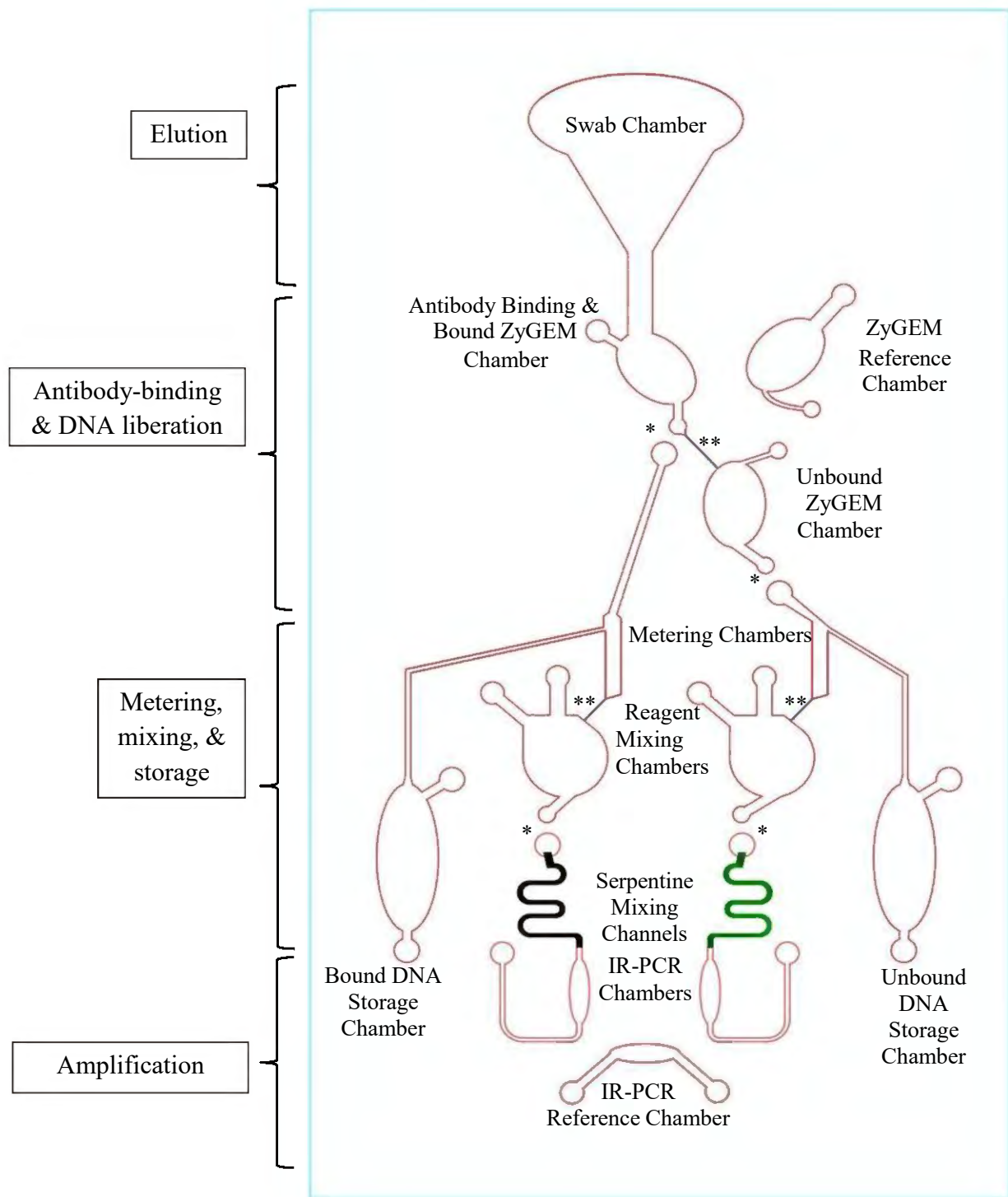


Figure 1. Initial sexual assault microchip. This microdevice utilizes an antibody-polystyrene bead complex to bind sperm cells in the antibody-binding chamber, which separates the sperm from non-sperm cells in a sexual assault sample. After a brief spin step, the bound and unbound cells are separated to the sperm and non-sperm ZyGEM chambers, respectively, and are processed in parallel. A * indicates a mechanical valve, while a ** indicates a burst valve.

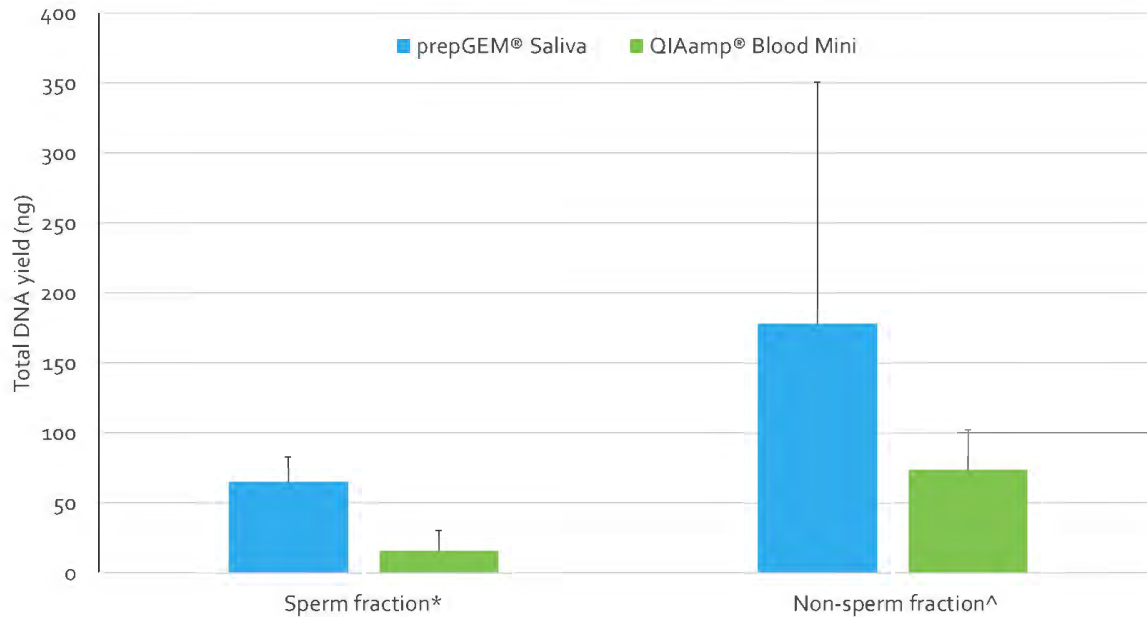


Figure 2. DNA yields obtained from differential DNA extractions using a modified *prepGEM*[®] method compared to a traditional differential method using QIAamp[®] DNA Blood Mini kit. *prepGEM*[®] resulted in a twofold increase in DNA in the non-sperm fraction, on average, versus the traditional method. More importantly, the *prepGEM*[®] modified method with added DTT resulted in a threefold increase in the DNA yield from the sperm fraction, on average, versus the traditional method. While not significant, these results show that the experimental modified differential *prepGEM*[®] method consistently improved the amount of DNA released and captured for amplification in both sperm- and non-sperm fractions thus indicating its suitability for integration into the sexual assault microdevice.

Table 1: Prospective antibodies reviewed for sperm-cell capture

Rank	Sperm Antibody	Species Specificity	Target Antigen Location	Expression Level
1	Intra-Acrosomal Protein Antibody (SP-10)*	Human, mice	Acrosome, sperm head	High
2	Sperm associated antigen (SPAG 8) (also referred to as Sperm membrane protein 1)*	Human	Acrosome and testis	High
3	Sperm Adhesion Molecule 1 (PH-20/ SPAM-1)*	Human, mice	Acrosome, epididymis, sperm head	High
4	A Kinase Anchoring Protein 3 (AKAP3)*	Human, mice, rat	Acrosome and tail of sperm, cytoplasm	High
5	Cysteine-rich secretory protein 2 (CRISP2)*	Human, mouse, rat, chicken, dog	Acrosomal cap, testis and epididymis	High
6	Motile sperm domain containing 3 (MOSPD3)*	Human, mice, rat	Distributed through the sperm, but concentrated on head and tail	High
7	Disintegrin and metalloproteinase domain-containing protein 2 (ADAM2)	Human, mouse, rat, chicken, dog	Sperm surface protein	High
8	Zona pellucida receptor protein 2 (ZP2)	Human	Acrosomal cap	High
9	Zona pellucida receptor protein (ZP1)	Human	Acrosomal cap-coded by the female helps bind sperm to egg	High
10	Sperm associated antigen 9 (SPAG9)	Human	Acrosomal cap-associated with infertility	High
11	Sperm agglutination antigen-1 SAGA-1	Human, mice	Epididymis and multiple locations along the sperm cell	High
12	Sperm acrosome membrane-associated protein (SPACA-1) (also called SAMP32)	Human, mice	Acrosome, sperm head, membrane protein localized in the equatorial segment of spermatozoa	High
13	Sperm associated antigen 6 (SPAG6)	Human	Tail-associated with infertility	High
14	Human epididymis-specific protein 5 (CD52)	Human, mouse, rat	Male reproductive track, specifically the epididymis	High
15	Anti-angiotensin-converting enzyme	Human, mouse, rat	Type-1 angiotensin II receptor on the spermatozoa located on tails, neck and mid-piece and flagellums of the sperm	High

*Antibodies tested in this project

Table 2: Flow Cytometry Results of Sperm-Specific Antibodies

Antibody	Positively stained sperm cells (%)
SPAG-8	19.7 ± 12.2
SP-10	14.08 ± 8.03
SPAM1/PH-20	74.18 ± 10.57
AKAP3	0.167 ± 1.801

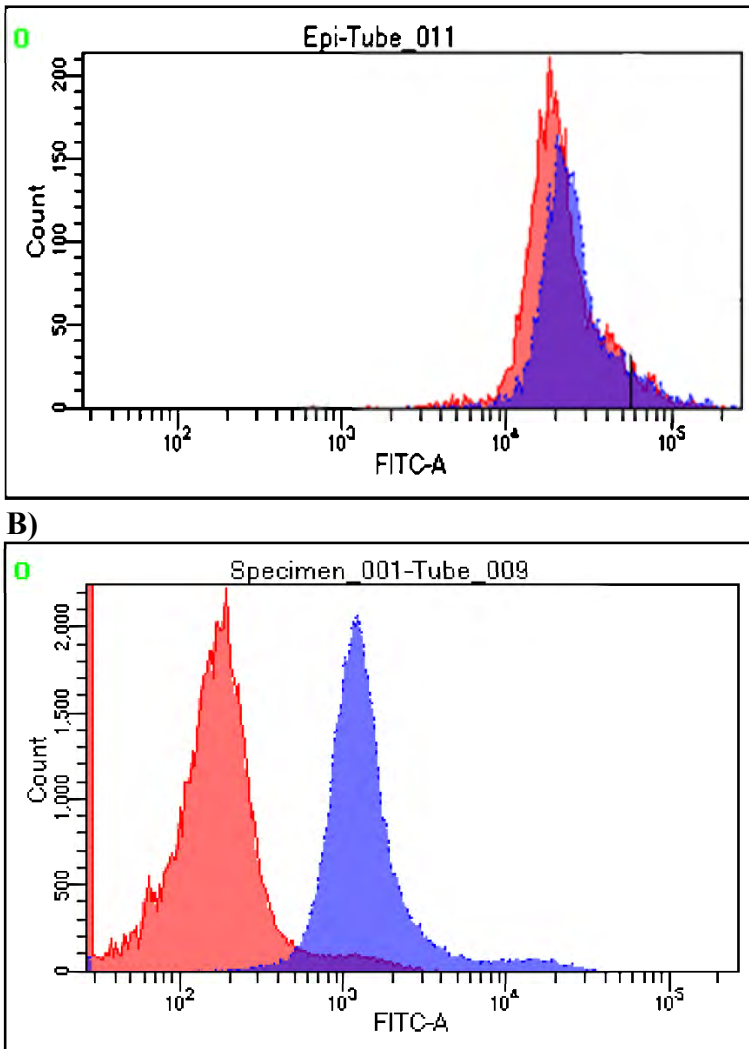


Figure 3. PH20 flow cytometry data showing positive cells within the gated population. (A) gated vaginal epithelial cells; (B) gated sperm cells. The isotype control is in red. The cells stained with the antibody for vaginal epithelial cells and for sperm cells are in blue. The x-axis represents the fluorescence intensity, and the y-axis is the number of events (cells) detected. The extreme forward shift seen in (B) indicates that the majority of sperm cells present are PH-20 positive.

Table 3: DNA Yields from Bead-mediated Antibody Sperm Cell Capture

Antibody	% of Total Semen DNA in Bound fraction
SPAG-8	70.09 ± 22.96
SPAM1/PH-20	37.66 ± 22.28
AKAP3	41.11 ± 18.04
MOSPD3	38.79 ± 33.98
CRISP2	52.61 ± 46.92
CK4	31.13 ± 9.00*

*Cytokeratin 4 antibody targets mucosal epithelial cells

Table 4. STR results showed SPAM1/PH20, AKAP3, and CK4 antibodies successfully enrich for male profiles in the bound fractions of semen-vaginal mixtures.

Male:female peak height ratios of tested antibodies		
Antibody Target	Semen-Vaginal Mixture Samples	
	Male:Female Ratio	
n=3	Bound Fraction	Unbound Fraction
SPAM-1/PH-20	10:1	14:1
AKAP3	9.6:1	6.5:1
SPAG8	6.1:1	10:1
CRISP2	4.0 :1	1.3:1
MOSPD3	1:2.1	1.7:1
CK4*	5.7:1	8.9:1

*CK4 targets mucosal epithelial cells, therefore this is the desired outcome

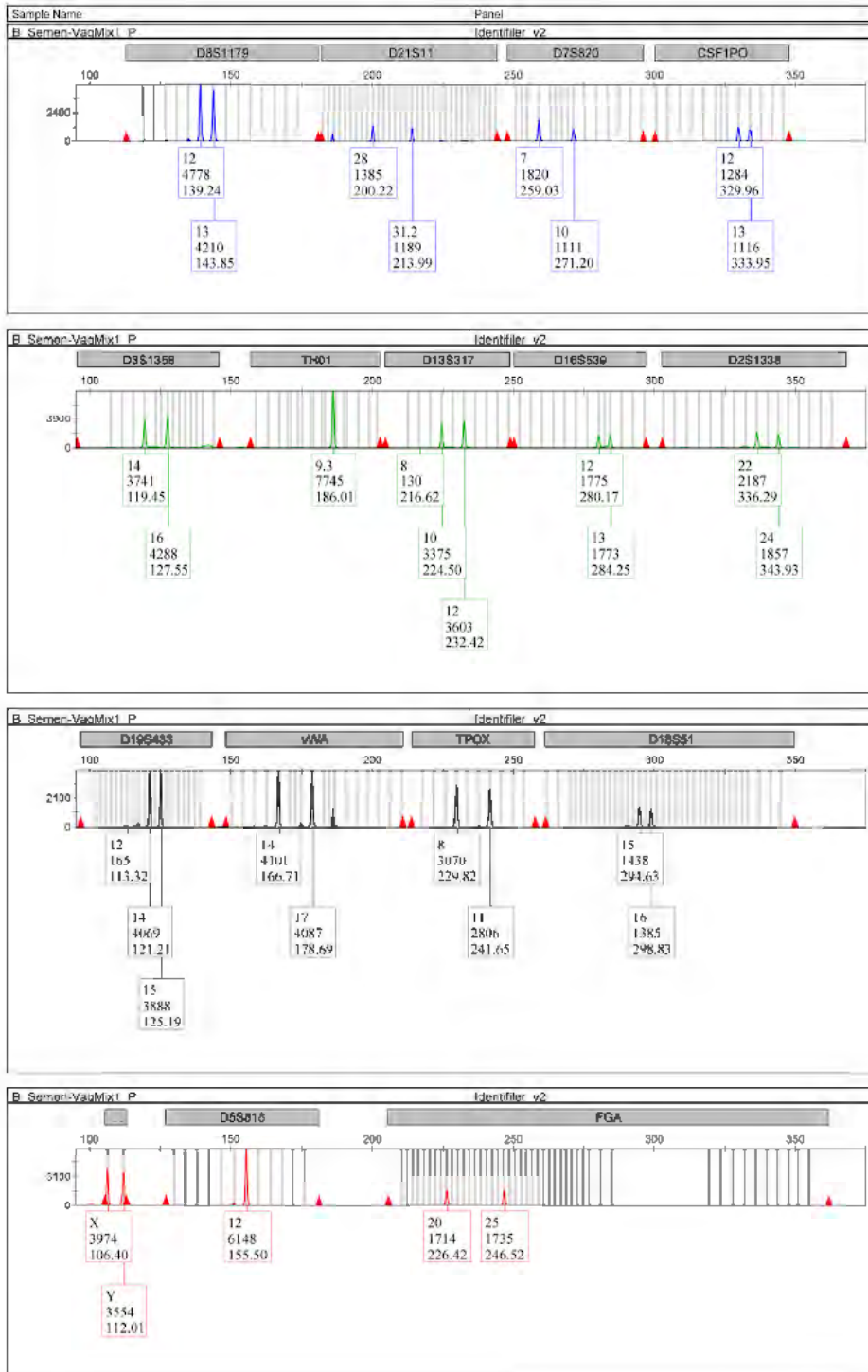


Figure 4. Electropherogram of a bound fraction of a semen: vaginal mixture sample using the antibody-bead binding mechanism with PH20. This shows a single-source male profile with the expected alleles.

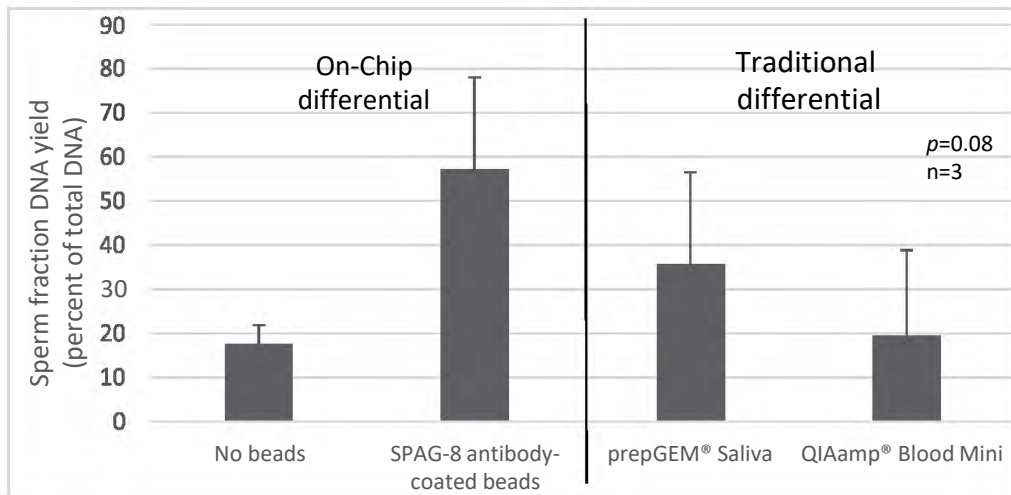


Figure 5. Percent of total semen sperm fraction DNA yield from the bead pellet using on-chip antibody-coated beads to capture sperm cells versus traditional differential methods. The SPAG-8 antibody-coated beads tested on-chip using the simple chip design and the modified *prepGEM*TM method yielded, on average, more sperm DNA in the bead pellet than the sperm fractions from traditional differential lysis and DNA purification methods. This data, along with the observed increase in total DNA yields using the antibody-mediated chip based method (data not shown), indicates that the SPAG-8 antibody-coated bead mechanism used on a microchip device is capable of more successfully binding, and thus, separating sperm cells than traditional differential methods.

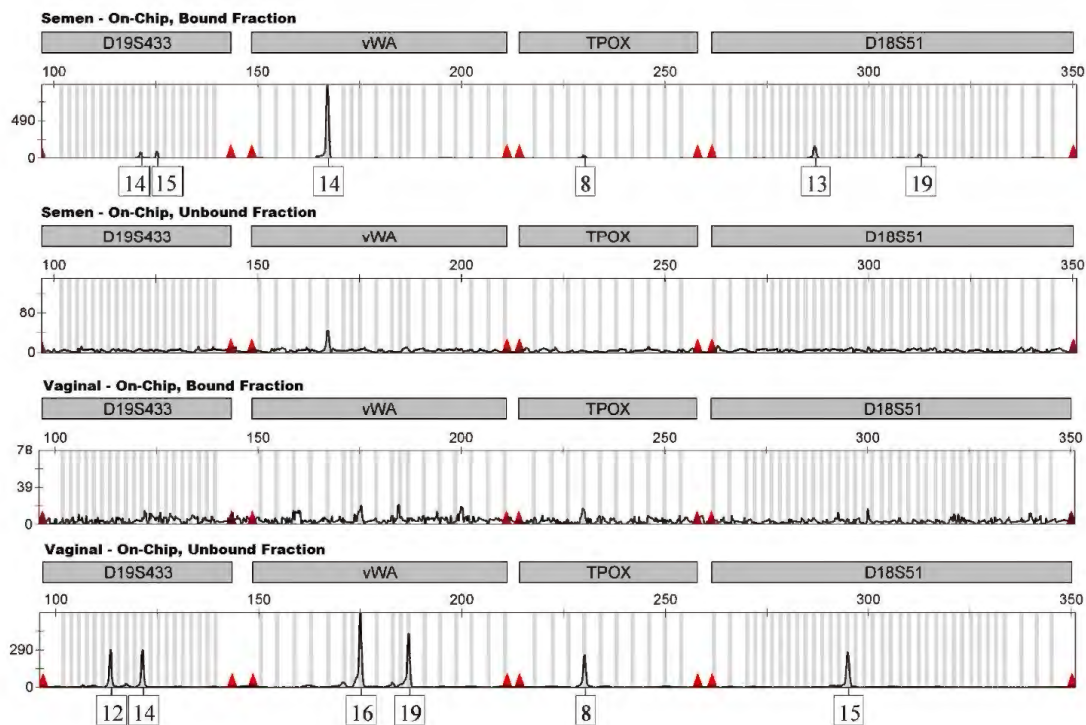


Figure 6. Electropherogram screenshots of the yellow channel for on-chip SPAG-8 semen and vaginal samples. The bound fraction completely enriched in the semen sample, while the unbound fraction completely enriched in the vaginal sample.

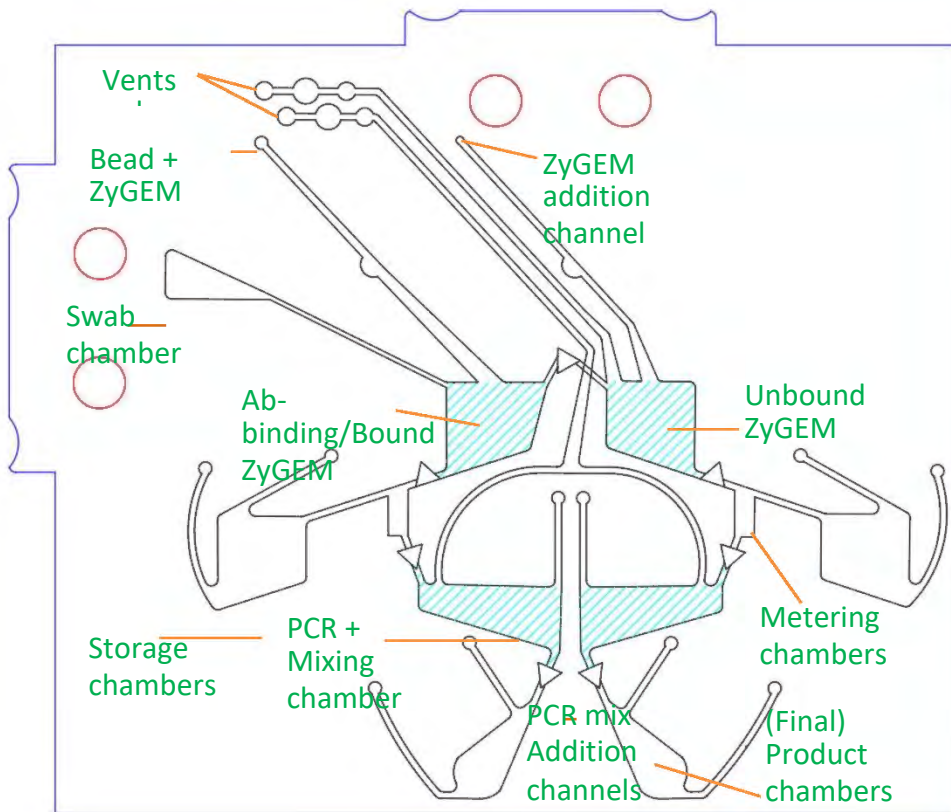


Figure 7. Sexual assault microdevice designed for new spin/heating platform. Workflow proceeds similarly to the device in Fig.1, with the major exception of laser/tap valves. These are denoted as small triangles located between the various chambers. The Peltier clamps down on the shaded chambers for ZyGEM and PCR heating.

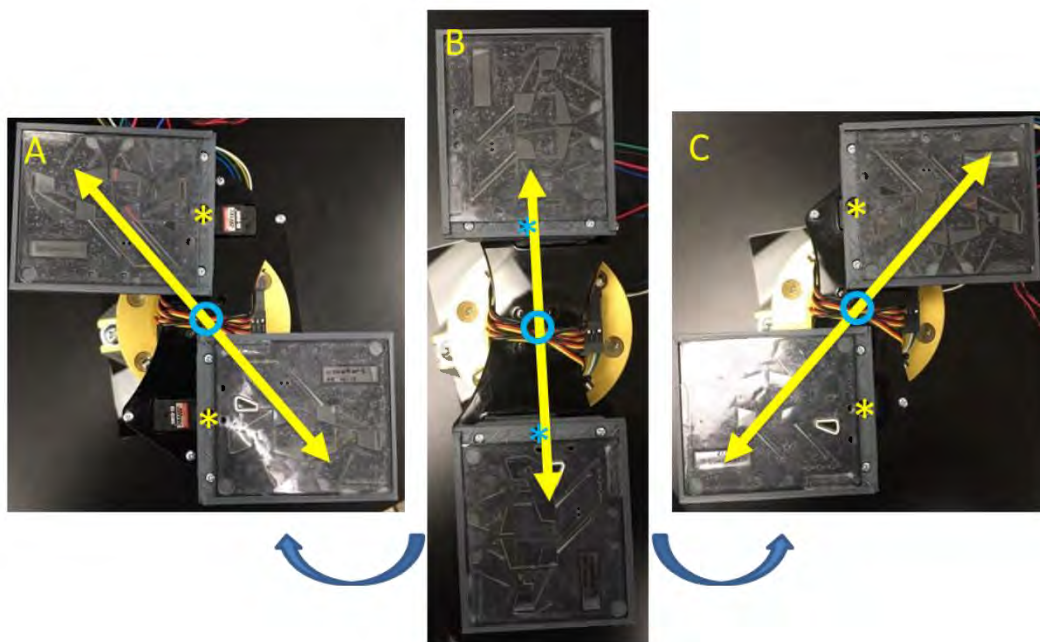


Figure 8. “Bird’s eye” view of sexual assault microdevice mounted on v3 spin platform, in “centered” (B), “left” (A), and “right” (C) positions. When the servos (*) rotate, the orientation of the microdevice relative to the center of rotation (blue circle) is altered. This allows flow direction about the chip (yellow arrows) to change. Note: servos can rotate to any position between A and C with resolution of 1°.

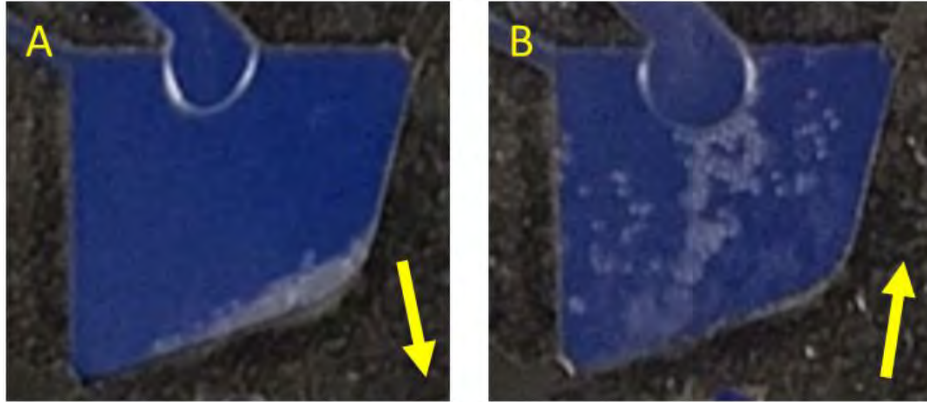


Figure 9. Close up view of bound chamber in a microdevice. When beads/cellular suspension are spun in one single direction, they are prone to “pelleting” against the “far wall” (A). When spin direction (yellow arrow) is altered via servo rotation, beads/cells can disperse throughout the chamber if spin speed is reduced (B).



Figure 10. Green channel electropherogram results of on-chip mixtures separated using SPAM1/PH-20 antibody. The bound fraction (top panel) showed a 142% enrichment for the male profile over the unbound fraction (bottom panel).

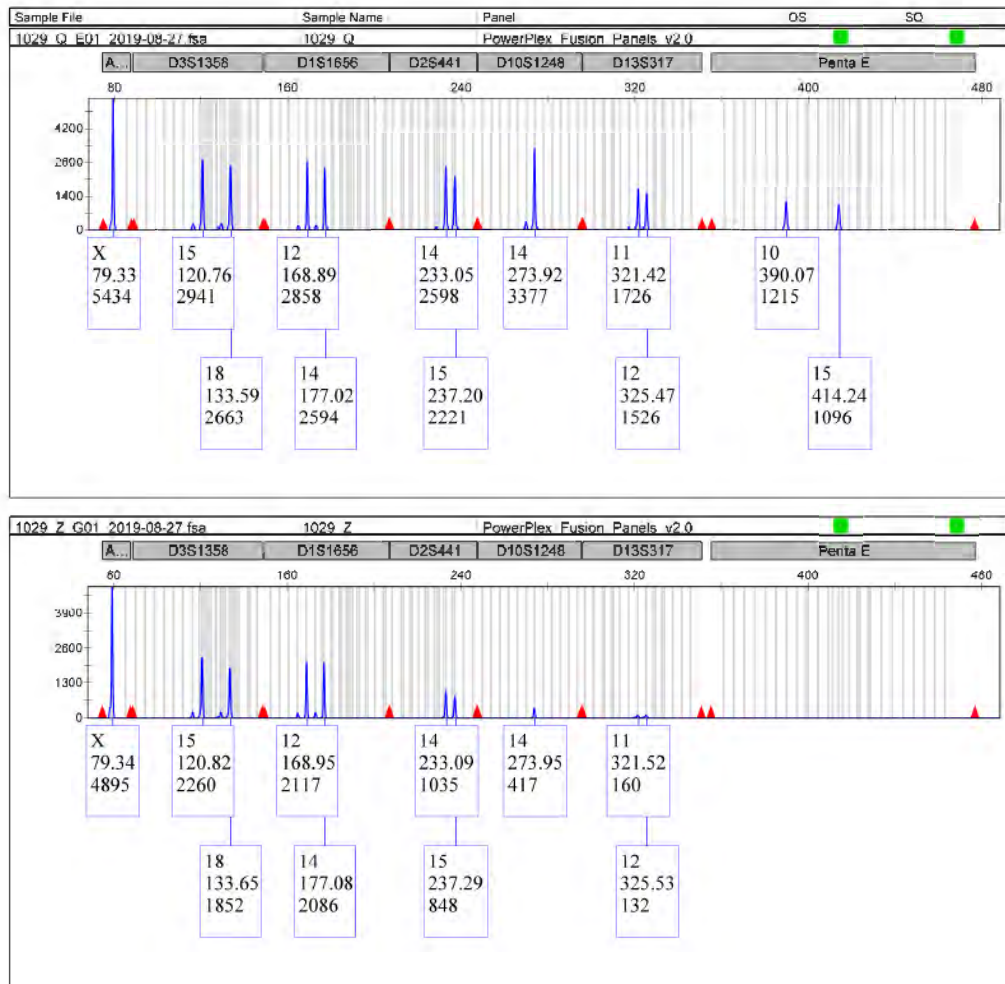


Figure 11. Blue channel electropherogram results of conventional Qiagen-amplified DNA (top panel) and on-chip forensicGEM-liberated DNA (bottom panel) when amplified with a custom, reduced-volume PCR chemistry and PowerPlex Fusion 5C primers. Overall, Qiagen-extracted DNA outperformed on-chip forensicGEM-liberated DNA in terms of alleles amplified, peak height, and peak balance.