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Abstract:

The purpose and scope of this project was to demonstrate the feasibility of a microfluidic-controlled array-based system for SNP-typing. Y-chromosome SNP (Y-SNP) markers that could be utilized for forensic applications were chosen as the model system for this feasibility demonstration. For this project, we developed a microfluidic-based PCR amplification sub-circuit and microarray that incorporated Y-SNP markers. A conventional PCR plus hybridization-based approach, as well as an allele-specific arrayed primer-extension method were used in this demonstration. We also established feasibility for automating nucleic acid sample preparation from oral and semen samples. Overall, Phase 1 established Akonni's gel drop microfluidic microarray platform as an inexpensive, flexible solution for forensic typing of Y-chromosome and other similar SNP markers. Performance criteria for this feasibility study included the capability of identifying ethnically relevant Y-SNP markers in a microarray, microfluidic-based platform; demonstrating ease of use and applicability of Akonni's sample extraction technology for neat semen and oral samples; demonstrating that an integrated sample processing cartridge can recover male DNA from neat semen samples; and verifying performance of an amplification subcircuit on forensic-type samples to demonstrate correct genotyping. The major deliverable for this phase is a final technical report supporting successful completion of this feasibility testing.

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EXECUTIVE SUMMARY

Akonni Biosystems, Inc. was awarded a grant from the National Institute of Justice, Office of Justice Programs, in September 2007 to determine the feasiblity of utilizing Akonni's patented gel element microarray technology (TruArray™) for the typing of SNPs for forensic applications. Y-chromosome SNPs were chosen as the model system for this demonstration. The ultimate design goal of the Akonni system is to incorporate state of the art microfluidic technology to automate processing from sample preparation to allele discrimination. In the field of DNA forensics, this technology could afford users with several key advantages including no sample splitting (the ability to analyze for tens to hundreds of SNP markers at one time), rapid processing (sample to answer in under 30 minutes), walk-away push-button technology, low-cost (affordable tests and instrumentation), self-contained cartridges (substantially reduced chances of cross-contamination), small-footprint (takes up very little bench space), portability (field or mobile lab use), and a flexible platform (amenable to both nucleic acid and protein targets).

Akonni Biosystems has an exclusive liscence to a mature gel element (or drop array) intellectual property portfolio originally developed at Argonne National Laboratory in collaboration with the Englehardt Institute of Molecular Biology. The portfolio is supported by >60 peer reviewed publications, 23 issued patents, and 10 patents pending for the manufacture and use of 3-dimensional gel element microarrays for protein, nucleic acid and on-chip PCR analyses. The fundamental difference between Akonni's 3-dimensional gel element arrays and other microarrays is that individual polymeric gel elements literally create an array of 3-dimensional "test tubes." Gel elements are covalently attached to a solid support (glass or plastic), but the microarray capture probes (either nucleic acid or protein) are covalently crosslinked to the polymer backbone instead of the two-dimensional substrate surface. Some of the practical benefits of a gel element array compared to competing microarray substrates include 10X increased probe binding capacity for greater signal to noise ratios and improved reaction rates; short oligonucleotide probes for improved assay specificity and lower production costs; and the ability to immobilize proteins (antibodies) for functional protein assays without protein denaturation or steric constraints typifying two-dimensional substrates.

Because SNPs are generally conserved through generations and have a low rate of mutation they can be useful in many forensic applications, but due to the lack of a uniform technology for their typing, SNPs have not played a large role in forensic testing to date. Since forensic typing laboratories of varying resources and capacity must make use of common forensic techniques; simplicity and reproducibility are key factors to consider. Technologies that are easily implemented, easy to standardize, and easy to quality control are therefore in demand. The cost of a technique, both in labor and reagents, also significantly affects its use and thus effectiveness. The development of a SNP-typing technology that combines sensitivity, discrimination power, analytical accuracy and robustness with operational simplicity, readiness for standardization, rapid turn-around time, multiplexing capacity, and low unit costs will be of substantial impact to this community. It is our assertion that the Akonni technology offers such advantages.

In Phase 1 of this study, we proposed to establish feasibility for automating nucleic acid sample preparation from oral and semen samples within a sample-preparation sub-circuit and to develop a multiplexed, PCR-based fluidic sub-circuit that incorporated Y-chromosome SNP (Y-SNP) markers. Later goals would be to pursue an integrated (sample-to-answer) TruArray™ test cartridge and an

advanced hardware design for forensic-type samples. The specific technical objectives and milestones for the Phase 1 project were to:

1. Develop a microfluidic, nucleic acid sample prep sub-circuit for oral and semen samples;
2. Develop a microfluidic, PCR-based array sub-circuit for Y chromosome SNP typing;
3. Verify the performance of the PCR sub-circuit through specificity tests; and
4. Verify the performance of the system on mock forensic samples to establish senstivitity limits.

Sample Preparation Sub-Circuits: The core technology for our sample preparation effort is a proprietary porous glass matrix that makes an excellent substrate for purifying nucleic acids. The uniform structure of the matrix provides predictable flow allowing the eluent to have similar fluid dynamics as the sample. This leads to a higher recovery during the elution process. The large porosity of the glass matrix allows very fast processing of viscous samples like semen and whole blood. Additionally, their rigid form factor adds to their versatility, allowing us to create a simple, easy to use pipet tip form factor. For inexpensive protocol development and optimization prior to automation on a liquid handling testbed, the TruTip was simply connected to a Rainin electronic pipette. The Akonni TruTip protocol was developed and compared to Qiagen's DNA mini kit and evaluated for sample prep efficiency using the real–time PCR using the Y-Quantifiler Human Male DNA Quantification kit from Applied Biosystems. Performance of the TruTip for neat semen and saliva samples was comparable if not better than commercially available kits tested, indicating the effectiveness of the TruTip protocol. In addition, TruTip was faster for semen extractions utilizing: 1) a 30 min incubation step compared to 60 min for Qiagen, and 2) a 4-min bind, wash, dry, and elution process compare to 15-20 min for Qiagen. Additionally, for saliva, the 4 minute (or less) total extraction yielded sufficient quantities of purified nucleic acid and only required room temperature reagents. These basic protocols then served as the starting points for integrated cartridge definition.

As protocols were established as described above, an automated sample preparation subcircuit was designed that consisted of a machined cartridge with inlets, outlets, and a receptacle for attaching a TruTip. The TruTip is disposable and replaced after each run. The machined cartridge and other liquid handling components that the sample comes in contact are presently reusable for rapid prototyping and protocol development. However, as the project progresses into the integrated cartridge in Phase II, all components and lines that the sample comes into contact with will be part of a one-time use disposable - sample will not come into contact with reusable components and lines. The pipet tip form factor will be maintained in the integrated cartridge that contains the sample preparation, PCR, and microarray subcircuits. The sample is introduced through a septum that seals after removal of the tip. A chaotroph is added by a Flow Control Station and mixed on the cartridge. The sample is discarded to waste. The Flow Contols Station contained a pump, multi-port valves, and the bulk reagents. The Flow Controls Station metered and moved fluids from the bulk reagents to the cartridge. Thus, protocols optimized using the Rainin pipet were converted to scripts on the Flow Contol Station. A self-sealing entry port for a pipettor allows easy introduction of the sample without the risk of opening caps, which are often a cause of contamination. Additionally, one-way valves make cartridge insertion and removal simple and easy without the risk of losing sample due to leakage after the process is complete.

Three semen samples were sequentially processed on the automated system. In the protocol, the semen sample was added to holding tube that also contains the other lysis components. The mixture was incubated for 30 min, then the automation script was initiated to perform the bind, wash, dry, and elution steps. The system was extensively flushed with water after each run, and some of the water flush after the third run was collected and saved to assess for carryover. Some carryover was expected since we did not implement harsh decontamination procedures since there was uncertainty on how harsh decontamination solutions would react to some of the materials, and the ultimate goal is to confine sample to the disposable. Results indicate that the three replicates had better CTs than that of the unprocessed sample, indicating the purification was successful. Carryover contamination was evident as indicated by a signal from the water flush. However, the CT was about 2 cycles higher (similar to the signal obtained from unprocessed) than that obtained from the elutions. Therefore, the carryover was not a significant contributor to the signals obtained from the eluted fractions. When a 10-fold higher sample of semen was used, the PCR signal from unprocessed semen was highly inhibited, whereas the processed semen sample displayed a strong positive PCR signal.

As more of the fluid lines are moved to the integrated cartridge to minimize dead volumes, and more dedicated single valves are introduced, the automation process will get more efficient and faster. In addition, we will implement a more stringent decontamination procedure to minimize carryover as we proceed forward with relatively expensive machined parts. No decontamination procedure will be required in the more finished cartridges that will be from inexpensive molded parts for one-time use disposables.

AS-APEX Array Approach: For proof of principle demonstration of SNP detection for this project, and in addition to basic hybridization array development, we tested the allele-specific arrayed primer extension approach (AS-APEX) with our patented gel drop microarrays. The normal convention is to amplify a PCR product, purify it from the nucleotides and primers, and fragment it using uracil DNA glycosylase. The fragmented product is applied to the array surface for hybridization to the immobilized array primers and subsequent single nucleotide incorporation of labeled dideoxynucleotides. In our case, we use un-purified and un-fragmented PCR product applied directly to the array surface for incorporation of labeled-dUTP along with the other non-labeled nucleotides. The results is a linear amplification scheme in which multiple fluorphors can be incorporated per target extension and the target can be recycled to interact with another un-extended primer. We have designed our primers so that a single primer is immobilized within the each gel element on the array and designed such that the ending 3′ base is at the SNP site. A separate primer is designed for each SNP to be detected. Extension by polymerase is inhibited if the 3′ nucleotide of the primer is mismatched to the target. In the presence of the correct target and matched 3’ base, polymerase incorporates fluorescent labeled nucleotides to produce the final signal. We have applied this strategy to the detection of Y-chromosome SNPs. In this study, three SNP regions were targeted, M2, defining most African Americans, M170, defining most Caucasians, and M175, defining most Asians.

The entire purification, amplification, AS-APEX process was performed on a semen sample to yield the first demonstration of “sample to answer” results. First, the semen sample was purified on the automated TruTip instrument as described previously. Then, the extracted DNA was amplified in a multiplex fashion on the MJ Research thermocycler or in separate single-plex assays on the Bladder thermocycler. Finally, the amplification product was successfully run through the AS-APEX assay and imaged on the Port Array 5000.

PCR and Microarray Sub-Circuits: Various iterations of subcircuits based on two basic approaches were designed and tested. The first type of subcircuit uses a single chamber to perform both PCR and microarray hybridization. The chamber contains a gel-spot microarray and also serves as the reaction chamber for thermal cycling. PCR reaction mix containing template is loaded into the chamber, thermal cycling is performed, and the product hybridizes to its respective immobilized probe(s). The second and more traditional approach utilizes two chambers, one for PCR and the other for microarray hybridization. In both approaches the reaction chamber is rigid to accommodate printing the gel-spot array.

Flow cell subcircuits containing either separate PCR and microarray chambers or a single chamber for coupled PCR/microarray hybridization were assembled to allow access ports for filling the chambers as well as geometries that allow the gel-drops to be contained with a reaction chamber. A plastic film bonds to the double-sided adhesive to provide a leak-tight seal. Double-sided adhesive can be susceptible to failure when thermal cycling using a conventional denaturing temperature (e.g. 95^{o}C), thus we evaluated alternative temperatures for denaturing. The flow cells are very reliable at this temperature and the PCR reactions appear to remain robust. The PCR chamber in the subcircuit was subjected to thermal cycling using the Akonni bladder thermal cycler, which was developed internally as a preferred means to interface thermal cycler heaters with the two types of subcircuits. The bladder thermal cycler alternatively gates two temperature-controlled fluids through pair bladders that tightly press against the opposite walls of the flat reaction chamber of the subcircuit. The bladder thermal cycler harbored a modular thermal cycling site consisting of two opposing bladder pouches within a plastic housing. The bladders, consisting of flexible and heat conductive material, contained inlet and outlet ports that connected with the fluid circulation system. Temperature-controlled fluid simultaneously entered and exited both bladders in the thermal cycling site. During operation, the temperature-controlled fluid filled and pressurized the bladders, which expanded and formed a pair of temperature-controlled surfaces that squeezed firmly against the sides of the reaction chamber. A reaction chamber in the form of a flat tube, flow cell, or subcircuit component of a cartridge is positioned between the two inflated bladders.

As a demonstration of implementing the bladder thermal cycler for the single chamber approach to perform coupled PCR and microarray hybridization, our well characterized biothreat microarray was used for the study. 1000 copies of *Bacillus anthracis* genomic DNA was mixed with PCR master mix and loaded into the PCR/microarray subcircuit. The subcircuit was positioned on the bladder thermal cycler and subjected to coupled thermal cycling and hybridization. The resultant microarray reader images from the *B. anthracis* DNA samples displayed positive signals only at the correct *B. anthracis* gel-drop probes. In addition, no positive signals were evident in coupled PCR and hybridization test using no target (negative control).

Conclusions and Implications: The goal of this Phase I project was to demonstrate the feasibility Akonni's sample preparation and microfluidic microarray subcomponents for analyzing SNP markers (e.g., Y-SNPs) using forensic-type samples. In this study, we were able to demonstrate successful in-cartridge DNA extraction from semen and oral (saliva) samples, successful multiplexed PCR amplification without the need for sample splitting (with various biochemistries and sub-circuit/array

configurations), and successful DNA analysis using a well-established microarray platform with forensic-type samples.

As described in this report, two sub-circuits were developed and tested: 1) the sample preparation sub-circuit and 2) the PCR/array sub-circuit. Studies for sample preparation included use of the TruTip as a stand-alone method for extraction and the incorporation of the TruTip form factor into an integrated Flow Control System. We observed that a TruTip™ protocol is more than sufficient to yield adequate quantities of human DNA from saliva (over 1ug amounts) which would be suitable for forensic analysis. This protocol does not require protease enzymes or heat in contrast to commercially available kits (e.g., Qiagen's DNA mini kit). It is extremely fast with a sample processing time of approximately 4 minutes or less and a minimal starting volume of liquid saliva (50ul). Overall, liquid saliva proved to be an easy sample type for collecting, storing and processing and therefore, easily amenable to forensic applications. Success using the automated microfluidic flow control system for sample preparation using semen samples as the starting material further demonstrates the feasibility of incorporating sample preparation sub-circuits within an end-to-end push button technology suitable for forensic applications. Follow-on studies will leverage off of these initial studies regarding testing additional sample matrixes (e.g., blood, semen, swabs, etc.) along with expanded sensitivity studies.

Multiplex asymmetric amplification with hybridization on TruArray containing probes targeted to different Y-chromosomal and autosomal SNPs showed applicability for correct identification of the SNPs of interest. The experiments for the multiplex PCR optimization, optimization of probe design (in order to get higher signals for some SNP probe pairs and balanced hybridization pattern), optimization of hybridization parameters, and sequencing of the regions of interest for uncharacterized samples can be explored for further utility. In addition, successful end-to-end processing of a forensic type sample was demonstrated using the TruTip sample extraction protocol for isolation human genomic DNA from semen followed by TruArray analysis using conventional PCR and hybridization. Although we succeeded in correctly genotyping the samples tested, the signal ratios and margin of error was lower compared to the AS-APEX assay. Therefore, APEX was the chosen method moving forward. During the testing process our assay revealed the correct typing of NIST sample 3, which had been incorrectly labeled by NIST as a deletion instead of an insertion for the M175 site. NIST verified that our result was correct and were pleased that we had brought the error to their attention. We have shown that the assay is highly sensitive with detection limits at 16 copies of genomic DNA. With further optimization we believe the assay could achieve the same single-copy detection capability as regular PCR. We optimized our reaction conditions and primer sequences to be highly selective with the ability to discriminate between single nucleotides in a high background of female DNA, a situation highly relevant in rape cases. The ratio range tested (0-10ng) was a result of material constraints of the available NIST samples. In future studies a wider range could be tested with higher concentration samples. Further studies could include increasing the ratio mixtures with higher concentrations of female DNA as well as testing 10ng or higher amounts of female-only DNA as a control.

Further optimization of the AS-APEX assay system will involve steps to further increase the SNP discrimination as well as to simplify the assay and minimize the reaction time. For example, we plan to examine different primer sequence modifications to increase the overall APEX signal and discrimination. We will also test approaches to eliminate the PCR amplification step by using whole genome amplification or combining the amplification reaction with the APEX assay for an "all-in-one" system. Both approaches would be tested in a flow-cell design, which allows for reduced contamination

within a sealed, thermal stable reaction chamber in contrast to the BioRad frame seal chambers which require opening to the environment for washing, drying and imaging. The eventual goal is to amplify the purified sample on the Bladder Thermal Cycler (in a multiplex assay), and perform the AS-APEX assay in a flow cell on the Bladder as well. Ultimately, each of these components will be integrated into one cartridge with a flow path between each step for a fully automated sample-to-answer system.

The next phases of this effort will build on the successful Phase I component and assay development tasks. Phase II will focus on defining protocols and assay chemistries, and packaging components and reagents into an integrated system for automated, sample-to-answer results. The system will consist of the instrument (i.e., liquid handling, Akonni Bladder Thermal Cycler, Akonni Reader, and cartridge docking station) and a disposable, integrated cartridge (i.e., Akonni TruTip, Akonni PCR and TruArray flow cell chambers, microfluidic circuits, and microfluidic valves). Emphasis will be placed on refining fluid paths (e.g., minimize the number of paths and path lengths), liquid handling and fluidic control (e.g. pumps, valve types and configurations), molding of the disposable plastic cartridge parts, developing an intuitive software and graphical user interface, and reducing the instrument footprint (approximately 2-3 cubic ft). Protocols and assays will be streamlined for minimum complexity, time, and cost. In addition, assay reagents (e.g., PCR, APEX) will be transitioned to a lyophilized format for long term storage and field deployment.

The successful development of an end-to-end SNP-typing cartridge would have direct (technical) implications for translating forensically important assays into a low-cost, user-friendly format. Given the fluidic process described herein, the same basic microfluidic format and manufacturing infrastructure could be applied directly to forensic serology with antigen/antibody testing; mRNA expression studies to determine tissue sources in biological material left at crime scenes; mitochondrial testing; ethnicity or phenotype determinations of semen donors in no-suspect rape cases; plant and/or non-human animal (i.e., dog or cat) genotype analysis; and many others. Given the portability of the TruArray™ system, its low cost and ease of use, the products of this research may also be translated to mobile crime units. This basic test system could aid in stain identification (to determine if multiple stains came from the same person or from different individuals), which could expedite the sample collection process at crime scenes. Finally, applications to forensic biodefense (e.g., anthrax or small pox testing) are today becoming a new and emerging application area that could benefit greatly from an integrated sample-to-answer rapid test platform.

MAIN REPORT

Introduction

Statement of the Problem and Relevance to Forensics

The need for rapid, accurate and reproducible SNP typing for forensic applications is increasing. Obvious application areas include Y-chromosome and mitochondrial DNA applications, but autosomal applications are also becoming more prevalent. Because SNPs are generally conserved through generations and have a low rate of mutation they can be useful in many forensic applications. These include inheritance or other family-linked cases, missing persons cases, identifying remains in mass casualties, or other instances where there is no reference sample [1]. Additionally, SNPs can be used to rapidly and accurately identify trace levels of DNA in complex mixtures [2, 3]. For example, high-density SNP assays can be used for complex mixtures to identify whether or not an individual's alleles are present without needing to know how many samples comprise the mixture or at what levels [2]. Since forensic typing laboratories of varying resources and capacity must make use of common forensic techniques; simplicity and reproducibility are key factors to consider. Technologies, such the one described herein, that are easily implemented, easy to standardize, and easy to quality control are therefore in demand. The cost of a technique, both in labor and reagents, also significantly affects its use and thus effectiveness, particularly in localities encumbered by limited financial resources.

SNPs are the most common polymorphisms in the human genome, occurring at an estimated frequency of once every 300-500 base pairs in non-coding regions and somewhat less frequently in protein coding and regulatory regions. Besides their high genomic frequency and binary nature, they are genetically stable, and are thus generally considered easy to genotype [1,4,5]. Also due to a SNPs small size and high frequency, smaller fragments of DNA (60-80bp in length) or degraded DNA samples can still be processed successfully [1,4,5,6]. Combining these advantages makes SNPs particularly appealing for forensics, but due to the lack of a uniform technology for their typing, SNPs have not played a large role in forensic testing to date.

To demonstrate the utility of the Akonni platform for typing SNP markers for forensic applications, Y-chromosome SNPs were chosen as the model system. Y-chromosome based tests, both those based on short tandem repeats (Y-STRs) or single nucleotide polymorphisms (Y-SNPs), can be utilized to help determine the identity of male donors in a rape kit sample, type azoospermic or vasectomized males, track paternal lineages, assist in missing persons cases, perform paternity testing, and can be used for genealogical research applications [7-11]. Technologies based on Y-SNPs have been widely used to study human migration patterns and genealogical histories, and may be applicable to forensics where trace amounts of male DNA may be present in a sample, or where the ethnicity of a male donor may be questioned [8-12].

Many methods exist today to test Y-SNPs, including allele-specific hybridization and primer extension [7,12], mini-sequencing [13], denaturing high performance chromatography and PCR-RFLP analysis (summarized in [10]). Butler [7] describes additional methods such as melting curve analysis, MALDI-TOF MS, real-time PCR analysis, microarrays, SNaPshot (Applied Biosystems), and Luminex bead hybridization methods (e.g., Marligen Biosciences). In general, many of these SNP typing technologies

do not rely on single-amplification schemes, or if they can accommodate multiplexed amplification, they are neither easily automatable nor low in cost. In some cases, such as real-time PCR they can also be limited by the number of probes that can be accommodated into their multiplex [3]. For these reasons, there may be interest and value in developing automated (sample-to-answer) test cartridges and protocols for forensically relevant SNP testing. Despite the fact that STR-based test systems have been put through the rigors of court-admissibility hearings and have emerged as standards in the industry, they are not without technical limitations, can be labor-intensive, and can require expensive equipment to run. Data analysis can also be an extremely time-consuming step. In some instances, such as identifying individuals in mixtures, individual labs can come up with different results based on individual methods and analysis [2]. Thus, there is a near-term need to augment and complement existing technologies with new technologies that can answer specific but relevant forensic questions, and do so in an operationally easy, low-cost, rapid and flexible manner.

The development of a SNP-typing technology that combines sensitivity, discrimination power, analytical accuracy and robustness with operational simplicity, readiness for standardization, rapid turn-around time, multiplexing capacity, and low unit costs will be of substantial impact to this community. The Akonni technology offers such advantages.

Background and Research Rationale

Akonni Gel Element Arrays. Akonni Biosystems has an exclusive liscence to a mature gel element (or drop array) intellectual property portfolio originally developed at Argonne National Laboratory in collaboration with the Englehardt Institute of Molecular Biology. The portfolio is supported by >60 peer reviewed publications, 23 issued patents, and 10 patents pending for the manufacture and use of 3-dimensional gel element microarrays for protein, nucleic acid and on-chip PCR analyses (e.g., [14-24]), with the basic technology now deployed in 12 government and academic laboratories around the country (including the Food and Drug Administration, U.S. Army, U.S. Air Force and the Naval Research Laboratory). The fundamental difference between Akonni's 3-dimensional gel element arrays and other microarrays is that individual polymeric gel elements literally create an array of 3-dimensional "test tubes" (Figures 1 and 2). Gel elements are covalently attached to a solid support (glass or plastic), but the microarray capture probes (either nucleic acid or protein) are covalently crosslinked to the polymer backbone instead of the two-dimensional substrate surface. Some of the practical benefits of a gel element array compared to competing microarray substrates include 10X increased probe binding capacity for greater signal to noise ratios and improved reaction rates; short oligonucleotide probes for improved assay specificity and lower production costs; and the ability to immobilize proteins (antibodies) for functional protein assays without protein denaturation or steric constraints typifying two-dimensional substrates.

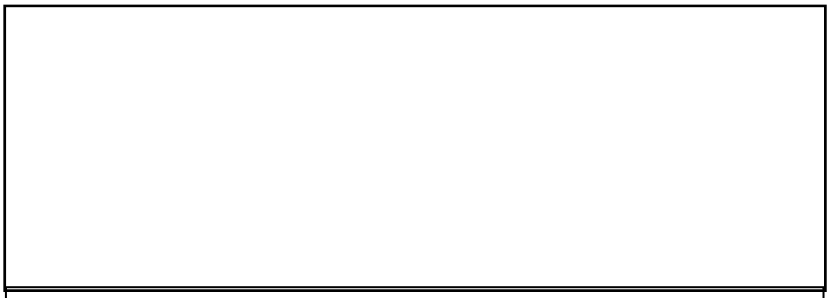


Figure 1. Akonni "gel drop" microarray platform. Probes are covalently attached to the polymer backbone rather than a solid substrate. Gel substrates are covalently linked to either a glass or plastic base and assembled into a test cartridge.

Overall Goals: The goal of this project was to determine the feasiblity of utilizing Akonni's patented gel element microarray technology (TruArray™) for the typing of SNPs in a forensic setting. Y-chromosome SNPs were chosen as the model system for this demonstration. The ultimate design goal of the Akonni system is to incorporate state of the art microfluidic technology to automate processing from sample preparation to allele discrimination, as illustrated in Figure 2. In the field of DNA forensics, this technology could afford users with several key advantages including no sample splitting (the ability to analyze for tens to hundreds of SNP markers at one time), rapid processing (sample to answer in under 30 minutes), walk-away push-button technology, low-cost (affordable tests and instrumentation), self-contained cartridges (substantially reduces chances of cross-contamination), small-footprint (takes up very little bench space), portability (field or mobile lab use), and a flexible platform (amenable to both nucleic acid and protein targets).

In Phase 1, we proposed to establish feasibility for automating nucleic acid sample preparation from oral and semen samples within a sample-preparation sub-circuit and to develop a multiplexed, PCR-based (MME-PCR, for example [25]) fluidic sub-circuit that incorporated Y-chromosome SNP (Y-SNP) markers. Later goals would be to pursue an integrated (sample-to-answer) TruArray™ test cartridge and an advanced hardware design for forensic-type samples. Future iterations of the basic TruArray™ cartridge and system could incorporate solutions to other forensically relevent questions (e.g., mtDNA tests, phenotype tests, antigen or tissue-specific mRNA tests). Ultimately, the resulting forensic TruArray™ system would include a portable reader device and self-contained, credit-card sized disposable tests that operate with a simple push of the button.

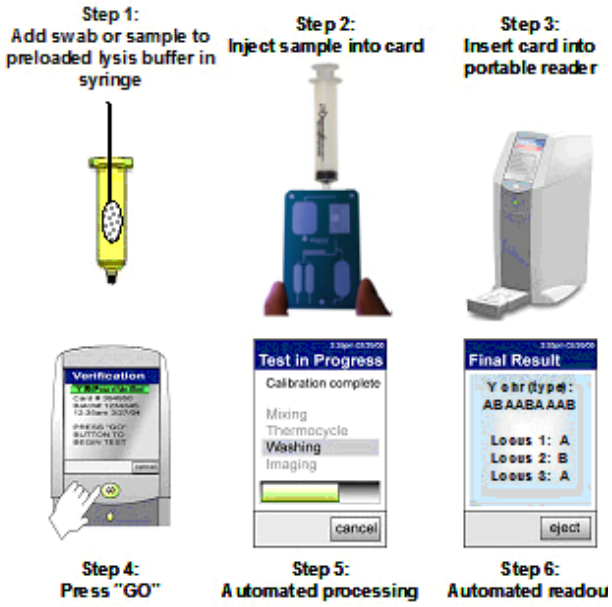


Figure 2. Concept of operations for Akonni's automated Y-SNP genotyping system.

Design and testing of a microarray containing Y-SNP markers: The primary step for array development was the down-selecting of exemplar SNPs for inclusion in the array. After discussions with our collaborators, we choose to explore feasibility around four ethnically-relevant and testable Y-SNP markers and one autosomal SNP marker in addition to Amelogenin (shown in Table 1) in the microarray test to be developed. One of the factors in this decision was to limit markers to those that could be tested with male samples currently available at NIST containing allelic polymorphisms in order to establish baseline performance specifications. The inclusion of an autosomal SNP, specifically GABRA2 [26], with an average heterozyogosity of 0.485, was encouraged by our collaborators to broaden the capabilities demonstration.

The allele-specific arrayed primer extension approach: For proof of principle demonstration of SNP detection, we tested the allele-specific arrayed primer extension approach (AS-APEX) with our patented gel drop microarrays. The APEX method has been previously used to discriminate single nucleotide differences from amplified PCR products on a microarray [27-31]. The normal convention is to amplify a PCR product, purify it from the nucleotides and primers, and fragment it using uracil DNA glycosylase. The fragmented product is applied to the array surface for hybridization to the immobilized array primers and subsequent single nucleotide incorporation of labeled dideoxynucleotides. In our case, we use un-purified and un-fragmented PCR product applied directly to the array surface for incorporation of labeled-dUTP along with the other non-labeled nucleotides. The results is a linear amplification scheme in which multiple fluorphors can be incorporated per target extension and the target can be recycled to interact with another un-extended primer. We have designed our primers so that a single primer is immobilized within the each gel element on the array and designed such that the ending 3′ base is at the SNP site. A separate primer is designed for each SNP to be detected. For instance, if there is a possibility for an A or a C at a certain SNP site, then a separate primer is designed for each, one ending in a 3′ A and one in a 3′ C. Extension by polymerase is inhibited if the 3′ nucleotide of the primer is mismatched to the target (e.g., see Figure 3). In the presence of the correct target and matched 3' base, polymerase incorporates fluorescent labeled nucleotides to produce the final signal. We have applied this strategy to the detection of Y-chromosome SNPs. In this study, three SNP regions were targeted, M2, defining most African Americans, M170, defining most Caucasians, and M175, defining most Asians.

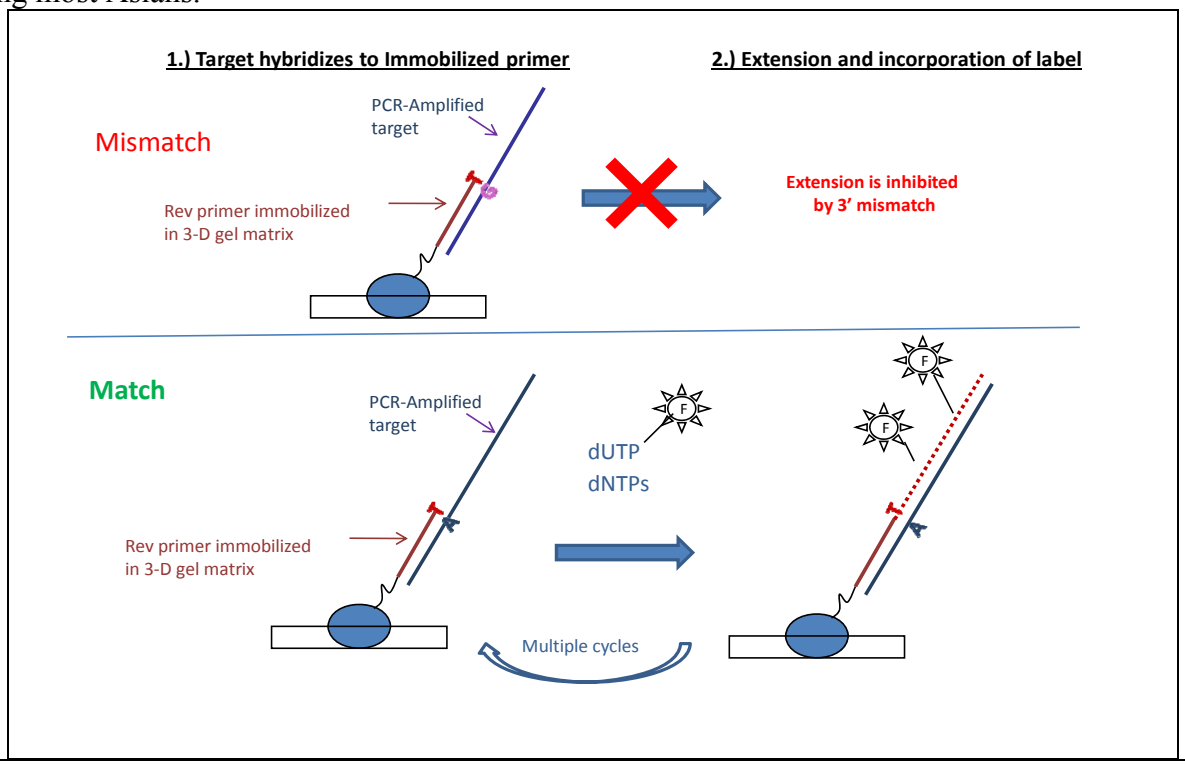
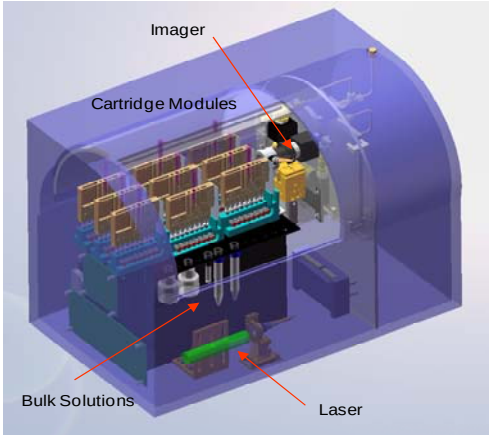


Figure 3: An illustration of the AS-APEX assay mechanism.

Fluidics and Integration: In consideration of future goals, we have designed an integrated fluidic cartridge that processes a sample and provides an answer using our gel spot microarray platform (Figure 4). Following introduction of the sample, the Flow Control Station (see Figure 13 later in this document) delivers the sample to a porous glass matrix as described in a later section. The Flow Control

Station interfaces with the cartridge to deliver the appropriate reagents. The waste is stored on the cartridge to prevent cross-over contamination from sample to sample. Following purification, the nucleic acid is eluted from the matrix and enters an elution chamber. The eluate is then combined with a PCR reagent and introduced into a PCR chamber and undergoes thermal cycling with our in-house developed Bladder thermal cycler. Following PCR, the product is mixed with a hybridization buffer which is then added to the microarray chamber for the readout. The product is then discarded to a waste chamber.



The sample preparation and Bladder Control thermocycler work described in Tasks 1 and 2 are designed to transition into a complete integrated cartridge for Phase II. The sample preparation binding matrix test fixture in Phase I represents the same form factor as envisioned for the integrated cartridge.

Figure 4. Concept of integrated cartridge

Specific Goals: In order to realize the goals of the project described in this report, and in collaboration with forensic experts from the Massachusetts State Police (MSP) Crime Laboratory, The National Institute of Standards and Technology (NIST), and Eric Buel of the Vermont State Police Crime Laboratory, the specific technical objectives and milestones for the Phase 1 project were to:

5. Develop a microfluidic, nucleic acid sample prep sub-circuit for oral and semen samples;
6. Develop a microfluidic, PCR-based array sub-circuit for Y chromosome SNP typing;
7. Verify the performance of the PCR sub-circuit through specificity tests; and
8. Verify the performance of the system on mock forensic samples to establish senstivitity limits.

Methods

M1. Sample Preparation.

The core technology for our sample preparation effort is a proprietary porous glass matrix that makes an excellent substrate for purifying nucleic acids. The uniform structure of the matrix provides predictable flow allowing the eluent to have similar fluid dynamics as the sample. This leads to a higher recovery during the elution process. The large porosity of the glass matrix allows very fast processing of viscous samples like semen and whole blood. Additionally, their rigid form factor adds to their versatility, allowing us to create a simple, easy to use pipet tip form factor. For protocol development and optimization, the glass matrix was fitted in a 2 ml pipet tip (called the TruTip) which was fluidically controlled by an electronic pipet.

M1.1. Manual TruTip Test Bed for Semen Extractions: For inexpensive protocol development and optimization prior to automation on a liquid handling testbed, the TruTip was simply connected to an electronic pipette (Rainin, Oakland, CA, US; Figure 5). The semen sample processed (labeled "Bob") was from a sperm bank (Xytex Corporation, Atlanta, GA, US) and represented a more challenging sample to process due to the presence of an egg yolk additive for long term storage.

The Akonni TruTip protocol was developed and compared to Qiagen's DNA mini kit (Qiagen, Valencia, CA, US) and evaluated for sample prep efficiency using the real–time PCR using the Y-Quantifiler Human Male DNA Quantification kit (Applied Biosystems, Foster City, CA, US). Three 1.5ml centrifuge tubes were labeled accordingly: Sample, Wash and Elution and 100µl of semen was added at desired dilution into the "Sample" tube. 80 µl of Qiagen AL Lysis Buffer, 20 µl of 1M DTT, and 10 µl of ProK was added to the sample. Samples were incubated at 55°C for 30 min and then 500 µl of 95% Ethanol was added to the mix. Using a TruTip, the sample was pipetted up and down (1 cycle) for 5 cycles total using a Rainin Electronic Pipettor (set to speed 5) in the sample tube. This step was followed by 5 cycles of washing using a pre-aliquoted 'Wash' tube. Air was then passed through TruTip to purge any remaining EtOH for 5 cycles. Once dried, the pipet was cycled 5 times with the elution buffer previously aliquoted (100ul of 10mM Tris-CL, pH 8.0 heated to 70°C) for 5 cycles. The tip was thrown away once the processing was completed and the eluate stored for later testing.

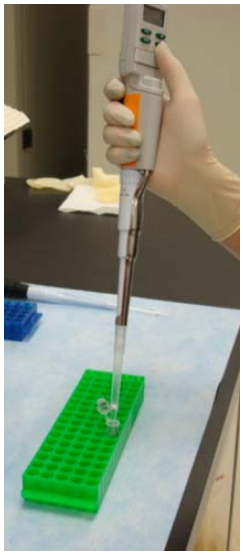


Figure 5: Manual TruTip Method. The TruTip component connected to an electronic pipette for controlled, repeatable fluid flow and ease of use.

A comparison of the Akonni TruTip protocol and Qiagen's DNA mini kit was conducted with a range of liquid semen dilutions. Both sample prep methods included Proteinase K and DTT with heated incubations for lysis (55°C) and elution (70°C). The purified human male DNAs were quantitated using Y-Quantifiler Human Male kit (AB) on the real-time LightCycler 480 (Roche Applied Science, Indianapolis, IN, US).

M1.2. Manual TruTip Test Bed for Saliva Extractions: The Akonni TruTip™ has been optimized for many sample types, e.g., bacterial and viral cultivars, sputum, nasal wash, semen and blood for either microbial or human nucleic acid of interest. Sample preparation experiments using human saliva as a sample type is of interest in forensic science as well as public health due to the non-invasive nature of sample collection along with easy sample handling. Our goal in these experiments was to evaluate various Akonni sample preparation protocols optimized for other sample types (blood, microbial cultivars, etc.) and identify an easy and timely procedure for obtaining adequate amounts of human DNA from saliva.

Human saliva, totaling 2ml, was collected from an anonymous volunteer using the Oragene-300 saliva collection kit per the manufacturer's protocol (DNA Genotek, Ottawa, Ontario, Canada). Using aliquots ranging from 50ul to 200ul from the 2ml of human saliva collected, sample preparation experiments were conducted comparing three different DNA extraction protocols: Akonni TruTip™ Easy, Akonni TruTip™ Blood and Qiagen DNA mini-kit.

The Akonni TruTip™ protocol for saliva (Akonni TruTip™ Easy) was developed and compared to the Akonni TruTip™ Blood protocol, and Qiagen's DNA Mini-Kit for Body Fluid (Qiagen, Valencia, CA, US). The Akonni Easy protocol contains a lysis buffer, without the use of protease and heat. The Qiagen DNA mini-kit contains a protease enzyme plus heated incubation (56°C for 10 minutes). The Akonni TruTip™ Blood Protocol is used to process the sample, followed by heated incubation. The Akonni TruTip™ attached to a Rainin automated pipettor (Rainin, Oakland, CA, US) was used for DNA binding and capture followed by wash steps and an elution step at optimal speed and volume settings. The Qiagen samples were processed according to the Qiagen DNA mini kit's recommended Body Fluid protocol.

The Akonni TruTip™ Easy Protocol consists of 4 main steps: Lysis & Binding, Wash, Dry and Elution. Four 1.5ml centrifuge tubes were labeled: Sample, Wash, Dry and Elution and 50ul to 200ul of saliva sample was added to the "Sample" tube. The sample was then brought up to 500ul with 1X PBS. 500µl of Lysis & Binding Buffer was added to the sample, briefly vortexed, and pulse spun. Using a large porosity TruTip™ (or LPT) the sample was pipetted up and down (1 cycle) for 5 cycles total using a Rainin Electronic Pipettor (set to speed 5) in the sample tube. This step was followed by 5 cycles of washing using a pre-aliquoted 'Wash' tube containing 500ul of 70% EtOH plus 500ul of 99% acetone. Air was then passed through the LPT, to purge any remaining buffer, for 5 cycles. Once dried, the electronic pipettor was cycled 5 times with the pre-aliquoted Elution Buffer (100ul of 10mM Tris-CL) for 5 cycles. The tip was thrown away once processing was completed and the eluate stored at 20°C for real-time PCR analysis.

Evaluation of sample preparation efficiency and nucleic acid recovery was performed using Applied Biosystems Human Quantifiler Real-time Assay (Applied Biosystems, Foster City, CA, US) on the Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN, US). Quantitation of the saliva DNA samples was obtained using a standard curve generated with the Human Quantifiler DNA standards included in AB's kit. For both the known standards and saliva DNA samples, 2ul of DNA was combined with AB's PCR reaction mix (12.5ul) and human primer (10.5ul) for a final reaction volume of 25ul. The thermal cycler program for saliva DNA samples and standards was as follows: initial denaturation of 95°C for 10 min; cycling of 95°C for 15s followed by 60°C for 1 min for 40 cycles total.

M1.3. Automated Sample Preparation Cartridge: Once the protocol was defined, the protocol was implemented into a test subcircuit or cartridge that accepted the modified pipet tip to achieve automation.

To reduce unwanted biomolecular (nucleic acid) adsorption to the cartridge surfaces we utilized a simple and short fluidic flow paths. The channels were larger than the typical microfluidic geometries. This scale reduced the surface-to-volume ratios and therefore reduced unwanted nucleic acid adsorption. Additionally, the simple flow path design improved the predictability of the device, which can often be compromised when controlling two-phase flow (liquids and air). The compliant nature of air often demands simplicity in flow design to ensure accurate and precise fluidic metering.

As part of the cartridge features we mininized, if not eliminated, possible losses due to imprecise fluidic control, biomolecular adsorption to materials due to high surface-to-volume ratios, and ease of use. When considering fluidic control methods, we decided on the Flow Control Station developed by Global

FIA because of its accurate and precise fluidic metering, robust components, and its proven capabilities used in the field of automated sample preparation devices (e.g., currently used on the Lawrence Livermore Autonomous Pathogen Detection System).

Another property of this device was ease of use, having several key features that makes this approach attractive compared to conventional sample preparation technologies. First, this device did not require centrifugation and thus eliminates the complexity associated with transferring samples from tubes to spin columns as well as simplified the instrumentation required. Additionally, a self-sealing entry port for a pipettor allowed easy introduction of the sample without the risk of opening caps, which are often a cause of contamination.

M2. Hybridization Array with Conventional PCR.

This section describes a method for detection of Y chromosome and autosomal SNPs using TruArrays based on conventional PCR and a separate hybridization. The method consists of multiplex asymmetric amplification of targets of interest and hybridization of the single-stranded amplicons on TruArrays containing a set of oligonucleotide probes targeting SNP regions of interest.

M2.1. Design of oligonucleotide probes and primers: Oligonucleotide probes targeting different SNPs (Tables 1 and 2) and PCR primers for multiplex amplification (Table 3) were designed using Oligo 6 software. Primers were designed to produce relatively short amplicons in order to minimize the influence of secondary structure on hybridization efficiency and avoid the necessity for fragmentation.

Table 1: List of targeted SNPs

SNP	Chrom.	Haplogroup	NCBI Ref ID	Alleles	Population Information
M2	Y	E3a	rs3893	A/G	Most African Americans
M170	Y	I	rs2032597	A/C	Common in Caucasians
M175	Y	O	rs2032678	-/TTCTC	Probable East Asia; later South Pacific
M269	Y	R1b1c	rs9786153	C/T	Most Caucasians
Amelogenin	X,Y	n/a	GenBank M55419	C(X)/T(Y)	For sex-typing purposes
GABRA2	4	n/a	rs279844	A/T	Autosomal SNP

M2.2. Synthesis of primers and oligonucleotide probes for TruArray: Oligonucleotide synthesis was carried out on an AB 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, US) at 1 μmol scale using commercial β-cyanoethyl phosphoramidites. Methacrylamido-modifier CPG was used as a solid phase for synthesis of oligonucleotides probes containing a methacrylic function at the 3'-ends. Oligonucleotides were cleaved from the CPG, protecting groups removed by standard procedures, and products purified by reverse phase HPLC. HPLC-purified oligonucleotides were evaporated to dryness (CentiVap concentrator, Labconco, Kansas City, MO, US) and reconstituted in 500 microliters of Milli-Q water for quantitation by UV adsorption (UV/VIS Spectrophotometer Lambda Bio 10, Perkin Elmer, Boston, MA, US). Thereafter, oligonucleotides were normalized in Milli-Q water to a final concentration of 2 mM and stored at -20ºC until use. 5'-Cy3 labeled oligonucleotide was synthesized by standard solid phase phosphoramidite chemistry using commercial Cy3™ phosphoramidite (Glen Research, Sterling, VA, US), and purified by RP HPLC after the deprotection procedure.

Table 2: TruArray probes for Y-SNP and autosomal SNP detection. Probes for naturally occurring alleles are highlighted with bold; SNPs are highlighted with red.

ID	Sequence	NCBI Reference ID:Position and Length
M2_A	**5'-TCCACAGATCTCATATTCAACCAT-3'**	**rs3893:168L24**
M2_G	**5'-TCCACAGATCTCACATTCAACCAT-3'**	**rs3893:168L24**
M2_T	5'-TCCACAGATCTCAAATTCAACCAT-3'	rs3893:168L24
M2_C	5'-TCCACAGATCTCAGATTCAACCAT-3'	rs3893:168L24
M170_A	**5'-CACACTGAAAAAAATGAACAATGAT-3'**	**rs2032597:317L25**
M170_C	**5'-CACACTGAAAAAAAGGAACAATGAT-3'**	**rs2032597:317L25**
M170_T	5'-CACACTGAAAAAAAAGAACAATGAT-3'	rs2032597:317L25
M170_G	5'-CACACTGAAAAAAACGAACAATGAT-3'	rs2032597:317L25
M175_D	**5'-GTTCATTCTTGAGAAGTGAGAAGG-3'**	**rs2032678:70L24**
M175_I	**5'-TTCTTGAGAAGAGAAGTGAGAAGG-3'**	**rs2032678:70L24**
M269_C1	**5'-TCAATTTACCAGGATTAACCAAAC-3'**	**rs9786153:190L24**
M269_C2	**5'-TTCAATTTACCAGGATTAACCAAAC-3'**	**rs9786153:190L25**
M269_T1	**5'-TCAATTTACCAGAATTAACCAAAC-3'**	**rs9786153:190L24**
M269_T2	**5'-TTCAATTTACCAGAATTAACCAAAC-3'**	**rs9786153:190L25**
M269_A	5'-TCAATTTACCAGTATTAACCAAAC-3'	rs9786153:190L24
M269_G	5'-TCAATTTACCAGCATTAACCAAAC-3'	rs9786153:190L24
Chr4_A	**5'-AGAGAGTTGTGAGTTTTAATATCTGG-3'**	**rs279844:289L26**
Chr4_T	**5'-AGAGAGTTGTGAGATTTAATATCTGG-3'**	**rs279844:289L26**
Chr4_G	5'-AGAGAGTTGTGAGCTTTAATATCTGG-3'	rs279844:289L26
Chr4_C	5'-AGAGAGTTGTGAGGTTTAATATCTGG-3'	rs279844:289L26
AmelX	**5'-CCACTTGAGAAACATCTGGGAT-3'**	**NC_000023.9:3498L22**
AmelY1	**5'-TGAGAAACCACTTTATTTGGGAT-3'**	**NC_000024.8:4106L23**
AmelY2	**5'-ACCACTTGAGAAACCACTTTATTTG-3'**	**NC_000024.8:4110L25**

Table 3: List of PCR primers used for amplification.

ID	Sequence	NCBI Reference ID:Position and Length	Amplicon ID and Length
M2F	5'-Cy3-GGCTCCCCTGTTTAAAAATGTAGG-3'	rs3893:110L24	M2: 107bp
M2R	5'-GGAAAATACAGCTCCCCCTTTATC-3'	rs3893:193L24	
M170F	5'-Cy3-TCAAATAATTGCAGCTCTTATTAAGTTATG-3'	rs2032597:239U30	M170: 140bp
M170R	5'-ACAAAAACAGGTCCTCATTTTACAGT-3'	rs2032597:353L26	
M175F	5'-Cy3-CAACTCAACTCCAGTGATTTAAACTCTC-3'	rs2032678:27U28	M175: 169bp (w/ del) 174bp (w/o del)
M175R	5'-CCATGTACTTTGTCCAATGCTGA-3'	rs2032678:174L23	
M269F	5'-Cy3-TGGATTCTGTTACATGGTATCACA-3'	rs9786153:146U24	M269: 165bp
M269R	5'-CCAAGGTGCTGGGATTACAC-3'	rs9786153:291L20	
Chr4F	5'-Cy3-TTACTTTTGATACAAAGGGTTTGC-3'	rs279844:175U24	Chr4: 191bp
Chr4R	5'-CAGAAGCTACTGGGATATTAATTAGTTC-3'	rs279844:338L28	
AmelF*	5'-Cy3-GGCACCCTGGTTATATCAACTTC-3'	NC_000023.9:3383U23 NC_000024.8:3991U23	AmelX: 195bp AmelY: 201bp
AmelR*	5'-GAGGCCAACCATCAGAGCTTA-3'	NC_000023.9:3557L21 NC_000024.8:4171L21	

*Note: For Amelogenin primers, positions for both X and Y chromosomes are given.

M2.3. Genomic DNA samples: NIST SRM 2395 Human Y-chromosome DNA Profiling Standard DNAs representing different haplogroups were used for amplification and analysis on TruArray. The CEZ sample was a 1cm X 1cm cutting from a neat semen stain on gauze processed using

the Qiagen DNA mini kit including Proteinase K and DTT with a 1hr incubation at 56°C. The male DNA from this sample was quantitated using AB's Y-Quantifiler Human Male Quantification kit with results of 2ng/ul.

M2.4. Fabrication of TruArrays: Oligonucleotides were dissolved in Akonni's acrylamide-based polymerization mixture and transferred to the wells of a 384-well microtitration plate. From the plate, each sample was spotted onto a microscope slide which had been chemically-modified to covalently bind with the polymerization mixture. The spotting was carried out using a 150 micron blunt tip pin affixed to a Genetix Q-Array high throughput robotic microarrayer. The polymerization and chemical attachment of the drops is accomplished using a UV source to initiate the polymerization under an inert atmosphere of argon gas. After polymerization, the chips were washed in 0.1 M PBS buffer followed by deionized water and then stored dry.

M2.5. PCR: PCR was carried out in 50 µl volumes containing 1 ng of genomic DNA, 400 nM (200 nM for symmetric PCR) of forward Cy3-labeled primer and 40-100 nM (200 nM for symmetric PCR) of reverse primer in 1×Qiagen Multiplex PCR mix (Qiagen, Valencia, CA, US). For multiplex PCR, primer concentrations were the same. Amplification was carried out in 24-well plates in Piko thermocycler (Finnzymes, Woburn, MA, US). Thermal cycling parameters included initial denaturing at 95°C for 15 min; 45 cycles of 30 s at 94°C, 90 s at 62°C, 30 s at 72°C; and a final extension at 72°C for 5 min. Primer concentrations were optimized during multiplexing and these results are described in the Results section of this report.

M2.6. Hybridization: All chemical reagents used for hybridization were from Fisher Scientific (Pittsburgh, PA, US) unless otherwise indicated. Hybridization of the PCR products on TruArrays was carried out in a buffer containing 1 M guanidine thiocyanate, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, and 0.2% bovine serum albumin (Pierce Biotechnology, Rockford, IL, US). Typically, 15 µl of PCR product was taken for hybridization. Hybridization was carried out using Grace Bio-Labs PC20 CoverWell Incubation chambers (Grace Bio-Labs, Bend, OR, US) in Boekel InSlide Out Hybridization Oven (Boekel Scientific, Feasterville, PA, US). Hybridization was carried out at 40°C for 3h. Following the hybridization, the chambers were removed and the slides with the arrays and the slides were placed into High-Throughput Wash Station (Telechem, Sunnyvale, CA, US) with 500 ml of wash solution containing 6×Sodium Saline Phosphate EDTA buffer and 0.005% sodium dodecyl sulfate for 2 minutes with stirring. The biochips were then briefly washed in MilliQ water and dried with a mild air stream.

M2.7. Acquiring fluorescent images from TruArrays and calculation of hybridization signals: Following hybridization, fluorescent signals from the arrays were acquired on a fluorescent microscope designed at Argonne National Laboratory (Argonne, IL US). Signals were processed using PortArray software (Aurora Photonics, Lake Barrington, IL, US). Fluorescent signals were normalized to background (average from same size blank areas containing no gel pads with probes). Only signals with S/B > 2 were considered for further analysis and ratio calculation.

M2.8. TruArray layout and description: The array contained a set of probes for detection of three mutations and one deletion on Y chromosome, detection and discrimination between Amelogenin genes from Y and X chromosomes, and detection of one mutation on Chromosome 4 (Figure 6; also see Table 2). The array contained probes targeted to naturally occurring alleles (highlighted with bold) and additional experimental control probes for non-existing alleles. In addition, probes for identification of

M269 mutation were present in two variants (M269C1/M269T1 and M269C2/M269T2). Analysis of ratios between hybridization signals in a pair of probes targeted for the mutation of interest allowed for SNP identification in the DNA sample under study due to differences in melting temperatures and hybridization efficiency between probes forming perfect duplexes and mismatched duplexes with a target amplicon. In addition, probes Amelogenin and Chromosome 4 represented controls for when only female DNA was present. On-chip controls were also included on this chip and included Cy3 beacons, hybridization control oligonuceotides and blank gel elements. The Cy3-labeled beacons are used for orientation purposes and to ensure that co-polymerization worked. The blank gel elements are present to assess background signals due to the gel matrix itself. The hybridization control is intended to demonstrate the success of the microarray hybridization itself, regardless of the success of sample preparation and amplification. The entire map area shown in Figure 6 was replicated in quadruplicate on each microarray for 4-16 pseudo-replicates for each control and 4 pseudo-replicates for each chromosomal probe.

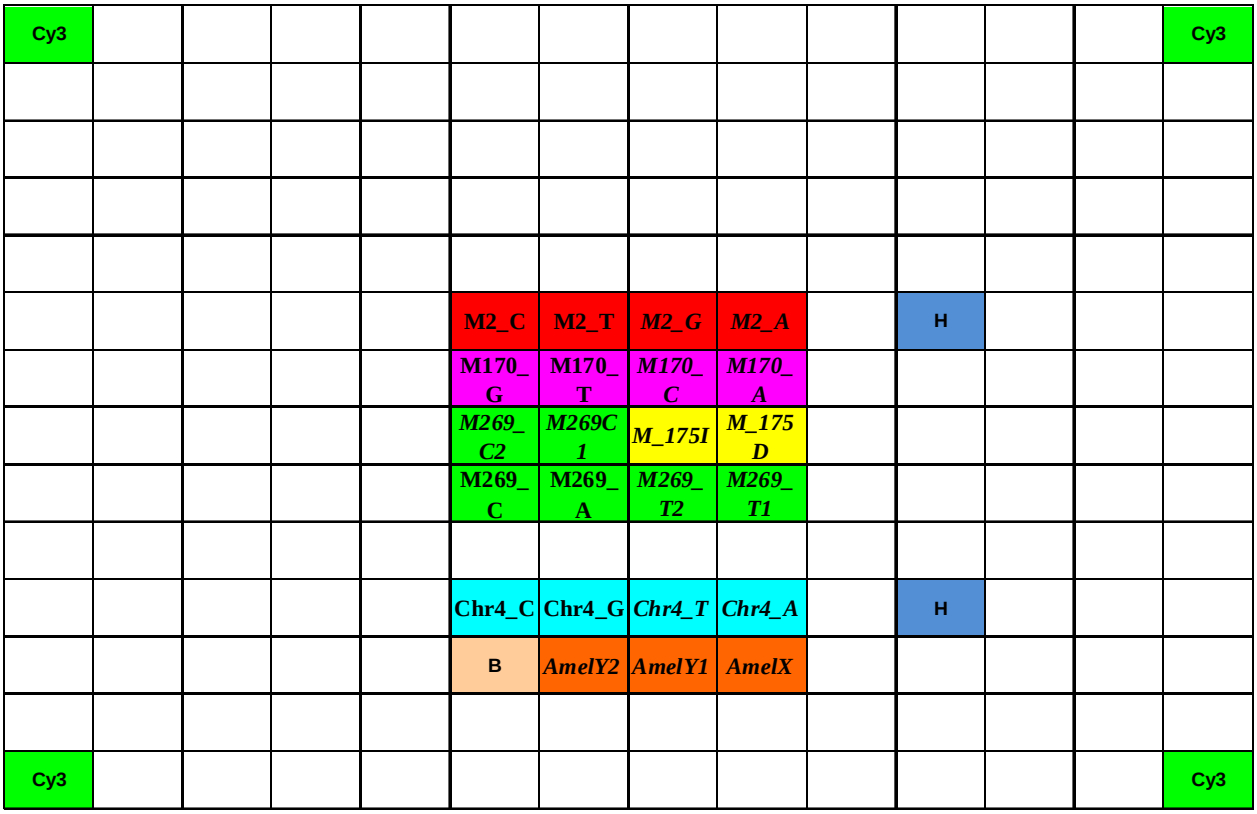


Figure 6: Schematic layout of TruArray for the SNP detection. Naturally occurring alleles are highlighted with italics. Controls include Cy3 beacons (Cy3), blank gel elements (B), and hybridization controls (H).

M3. Allele-Specific Arrayed Primer Extension.

In this section, we describe the approach of using allele-specific arrayed primer extension for SNP determination. SNP-specific primers are located within each gel element and a DNA polymerase extends preferentially off of the primer annealed to the target with no mismatches and incorporates

labeled dUTP (Alexa-546 dUTP) among other non-labeled nucleotides. The ratio of fluorescence signal from one allele specific primer to another is compared. Similar to the hybridization approach, any signal above 2 was considered to be positive for that SNP allele.

M3.1. Probe and array design: For consistency the reverse primer for each SNP site was the one immobilized within the gel element. These primers were synthesized and copolymerized in the gel elements as previously described in Section M2. These primers were designed to be 20-23 bases in length with a salt-adjusted Tm of roughly 60°C, and the 3′ base of the primer is the SNP site. A list of these primers is presented in Table 4. Different primer lengths were tested for optimal signal differential between the two alleles. Three concentrations of immobilized primers were printed on a single array: 10μM, 100μM, and 200μM.

M3.2. AS-APEX procedure: Briefly, for a 25μL reaction, 2.5μL of amplified target (from a multiplex PCR amplification with 1ng DNA target as described in M2) was added to a master mix consisting of 1X ThermoPol Buffer (NEB) , 0.6mg/mL BSA (Fisher), 2mM MgSO4 (amount added in addition to buffer, NEB), 10μM dATP/dCTP/dGTP (NEB), 9μM dTTP (NEB), 1μM Alexa-546-dUTP (Invitrogen), 5U Deep Vent Exo- polymerase (NEB) and 2.5U Inorganic Pyrophosphatase (NEB). The sample was applied to the APEX chamber formed by a 25μL Biorad frame seal (BioRad) surrounding the gel element array on a glass slide. A coverslip was applied to seal the chamber. The slide was placed in a slide adapter tower of an MJ Research PTC-200 thermocycler set to the following program (96.5°C for 2 min, 35 cycles of 94°C for 30 sec, 62.5°C for 1 min 15 sec, and 72°C for 5 min). After thermocycling, the coverslip and frame seal were removed from the slide using tweezers. The slide was then washed in buffer (1xSSPE + 0.01% Triton) for 5 minutes, rinsed in ROI water and dried with a mild air stream. Images of the array were captured using the PortArray 5000 as described previously. Exposure time was normally 2 seconds.

M3.3. Assay optimization: Initial testing of the AS-APEX assay was performed using single-plex products amplified on the MJ Research PCT-200 Tetrad. The cycling program was optimized to a two-temperature program with an effective annealing/extension temperature of ~66°C (equating to a setting of 64.5°C on the instrument). Once the optimal conditions were determined for the single-plex APEX assay, the multiplexed products were tested and demonstrated to also yield significant discrimination between the different SNP alleles. The optimized AS-APEX conditions for single-plex targets were then tested on multiplexed amplification products. Using purified Y-chromosomal DNA samples (1ng) with various genotypes obtained from NIST, each of the three targets (M2, M170, and M175) were amplified simultaneously in a multiplex reaction using the same conditions as in the single-plex amplification, but with multiple primers in the same reaction. Two and a half microliters of this amplification product was used in the 25μL AS-APEX reaction as described previously. The post-assay fluorescence images were then analyzed for relative fluorescence of the extended allele-specific SNP primers. Further optimization of the arrays involved examining the immobilized primer length and concentration. The arrays were printed at three concentrations of immobilized primer, 10μM, 100μM, and 200μM.

Table 4: TruArray Primers. Primers for TruArray for Y-SNP detection using AS-APEX (optimized probes used for the down-selected v2.1 array are shown in blue; SNPs are shown in red)

SNP Site	Oligo name	Sequence 5'-3'	Length
M2			
	M2_ASE_F_G_22	5'-Met-C18-CT TTA TCC TCC ACA GAT CTC AC-3'	22
	M2_ASE_F_G_21	5'-Met-C18-T TTA TCC TCC ACA GAT CTC AC-3'	21
	M2_ASE_F_G_20	5'-Met-C18- TTA TCC TCC ACA GAT CTC AC-3'	20
	M2_ASE_F_A_23	5'-Met_C18-CCT TTA TCC TCC ACA GAT CTC AT-3'	23
	M2_ASE_F_A_22	5'-Met_C18-CT TTA TCC TCC ACA GAT CTC AT-3'	22
	M2_ASE_F_A_21	5'-Met_C18-T TTA TCC TCC ACA GAT CTC AT-3'	21
M170			
	M170_ASE_F_C_22	5'-Met-C18-C ACA ACC CAC ACT GAA AAA AAG-3'	22
	M170_ASE_F_C_21	5'-Met-C18- ACA ACC CAC ACT GAA AAA AAG-3'	21
	M170_ASE_F_A_23	5'-Met-C18-AC ACA ACC CAC ACT GAA AAA AAT-3'	23
	M170_ASE_F_A_22	5'-Met-C18-C ACA ACC CAC ACT GAA AAA AAT-3'	22
M175			
	M175_D_F_1	5'-Met-C18-CT TGA GAA GTG AGA AGG CAT G-3'	21
	M175_D_F_2	5'-Met-C18-TCT GTT CAT TCT TGA GAA GTG AG-3'	23
	M175_I_F_1	5'-Met-C18-TCT TGA GAA GAG AAG TGA GAA G-3'	22
	M175_I_F_2	5'-Met-C18-TCT GTT CAT TCT TGA GAA GAG AA-3'	23

M3.4. Sample-to-answer demonstration: The entire purification, amplification, AS-APEX process was performed on a semen sample to yield the first demonstration of "sample to answer" results. First, the semen sample was purified on the automated TruTip instrument as described in M1. Then, the extracted DNA was amplified in a multiplex fashion on the MJ Research thermocycler or in separate single-plex assays on the Bladder thermocycler (see Figure 29 later in this document). Finally, the amplification product was run through the AS-APEX assay and imaged on the Port Array 5000.

M4. PCR and Microarray Sub-Circuit.

Flow cell subcircuits containing either separate PCR and microarray chambers or a single chamber for coupled PCR/microarray hybridization were assembled by bonding a double sided adhesive to a glass slide printed with a gel-drop microarray. Patterns were cut with a Universal Laser (Versa Laser VLS 3.5) to allow access ports for filling the chambers as well as geometries that allow the gel-drops to be contained with a rectangular reaction chamber. A plastic film bonds to the double-sided adhesive to provide a leak-tight seal. The PCR chamber in the subcircuit was subjected to thermal cycling using the Akonni bladder thermal cycler described below.

The bladder thermal cycler harbored a modular thermal cycling site consisting of two opposing bladder pouches within a plastic housing. The bladders, consisting of flexible and heat conductive material, contained inlet and outlet ports that connected with the fluid circulation system (see below).

Temperature-controlled fluid simultaneously entered and exited both bladders in the thermal cycling site. During operation, the temperature-controlled fluid filled and pressurized the bladders, which expanded and formed a pair of temperature-controlled surfaces that squeezed firmly against the sides of the reaction chamber. A reaction chamber in the form of a flat tube, flow cell, or subcircuit component of a cartridge is positioned between the two inflated bladders.

The dual-circulation system on the bladder thermal cycler included two in-line heat exchange blocks with cartridge heaters, two pumps, three three-way valves, and a bladder thermal cycling site. A PCR reaction chamber within the subcircuit was positioned in the interior space at the bladder thermal cycling site. Temperature-controlled fluid circulated through the bladder pair via a first circulating loop that included heat exchange block 1, pump 1, three-way valves 1 and 3. A second circulating loop included heat exchange block 2, pump 2, and three-way valves 2 and 3. Coordinated opening and closing of valves 1, 2, and 3 directed the temperature controlled fluid from the temperature zone 1 (kept at a temperature of, for example, 80-105ºC) to the temperature zone 2 (kept at a temperature of, for example, 50-70ºC) to flow through the bladder pair while the pumps 1 and 2 continued in one direction and re-circulated the fluid in the respective zone (see Figure 30 in Section R4.).

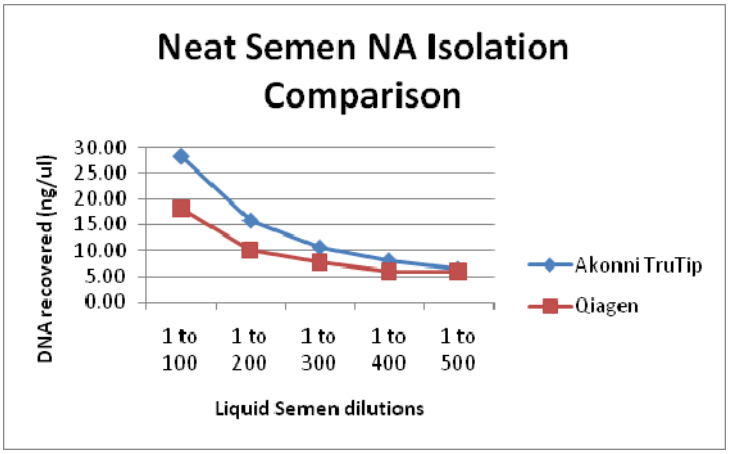


Figure 7: Liquid semen dilutions ranging from 1:100 to 1:500 were processed by both the Akonni TruTip and Qiagen extraction methods. For each, the sample input was 100ul and included 10ul of Pro K 20mg/ml, 20ul of 1M DTT and a 1hr incubation at 55C. Real-time PCR using the Y-Quantifiler Human Male Quantification kit (AB) was performed on the LightCycler 480 (Roche; input sample 2ul). The thermal cycling protocol was described previously.

Results

R1. Sample Preparation.

R1.1. Semen Extractions with Manual TruTip Method: Comparisons of Akonni's TruTip (operated by Rainin Electronic Pipettor) compared to Qiagen methods indicated that the TruTip performed as well or better than the Qiagen method (Figure 7).
Quantitative real-time PCR results from semen processed by TruTip and Qiagen protocols are also shown in Figure 8. Both methods gave similar results, indicating the effectiveness of the TruTip protocol. In addition, TruTip was faster, utilizing: 1) a 30 min incubation step compared to 60 min for Qiagen, and 2) a 4-min bind, wash, dry, and elution process compare to 15-20 min for Qiagen.

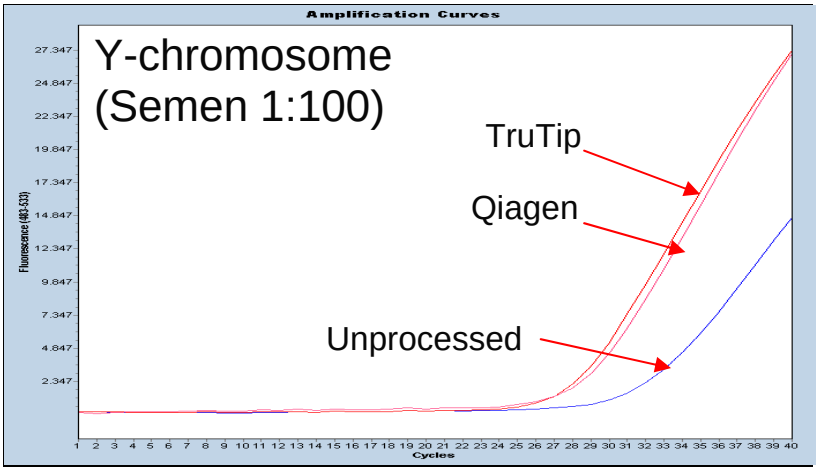


Figure 8. The TruTip device provides the same sample preparation performance as the commercial Qiagen kit. Incubation in the AL lysis buffer using the TruTip and Qiagen kit was performed for 30 and 60 min, respectively.

R1.2. Saliva Extractions with Manual TruTip Method: Figure 9 below displays the average DNA concentrations (ng/ul) recovered from three replicate saliva samples per extraction protocol tested. The final elution buffer for both the Akonni TruTip™ Easy and Akonni TruTip™ Blood was 1mM NaOH. Qiagen's elution buffer (AE) was used per the manufacturer's instructions. Quantitation was performed using the AB Human Quantifiler real-time assay and the Roche LightCycler 480 as described in Materials and Methods. The results are: 46.27ng/ul for Qiagen, 59.1ng/ul for the Akonni Blood protocol and 12.27ng/ul for the Akonni Easy protocol. The average DNA concentrations were converted to total Average DNA yield based on the 100ul elution volumes recovered and resulted as follows: 4.6ug for Qiagen, 6ug for Akonni Blood protocol and 1.23ug for Akonni Easy protocol.

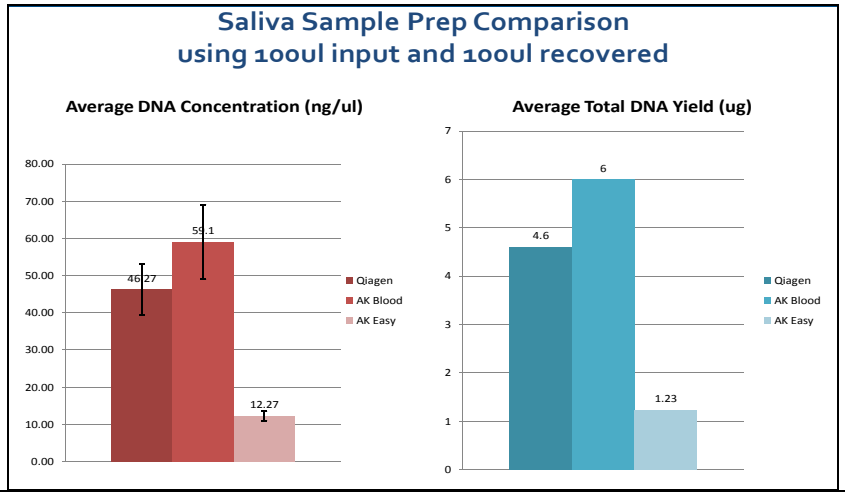


Figure 9: Average DNA concentrations for 100ul of saliva sample input.

Real-time PCR curves for various saliva sample volumes with different types of elution buffers (1mM NaOH versus heated 10mM Tris-HCL) processed using only the Akonni Easy protocol are shown in Figure 10. Sample volumes of 50ul and 200ul were extracted to obtain a range for adequate DNA recovery. Different elution buffers were tested for optimal long-term storage of DNA and ease of

processing. Saliva sample input volumes using 50ul and 200ul gave DNA concentrations ranging from 20.8ng/ul to 171ng/ul, respectively. The NaOH elution buffer resulted in lower DNA concentrations (20.8ng/ul) than the heated Tris-HCl elution buffer (33.1ng/ul); however, it was the opposite for the 200ul sample inputs (171ng/ul for NaOH and 141ng/ul for Tris-HCl). Slight inhibition of the real-time PCR curve was noted for the 200ul sample input volumes.

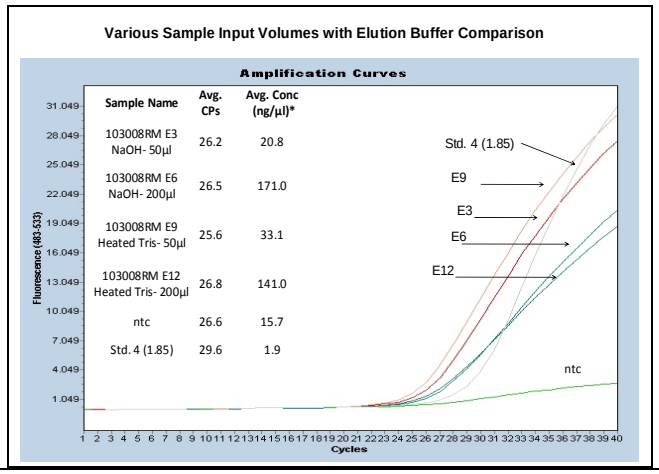


Figure 10: Saliva sample input volumes using 50ul and 200ul volumes.

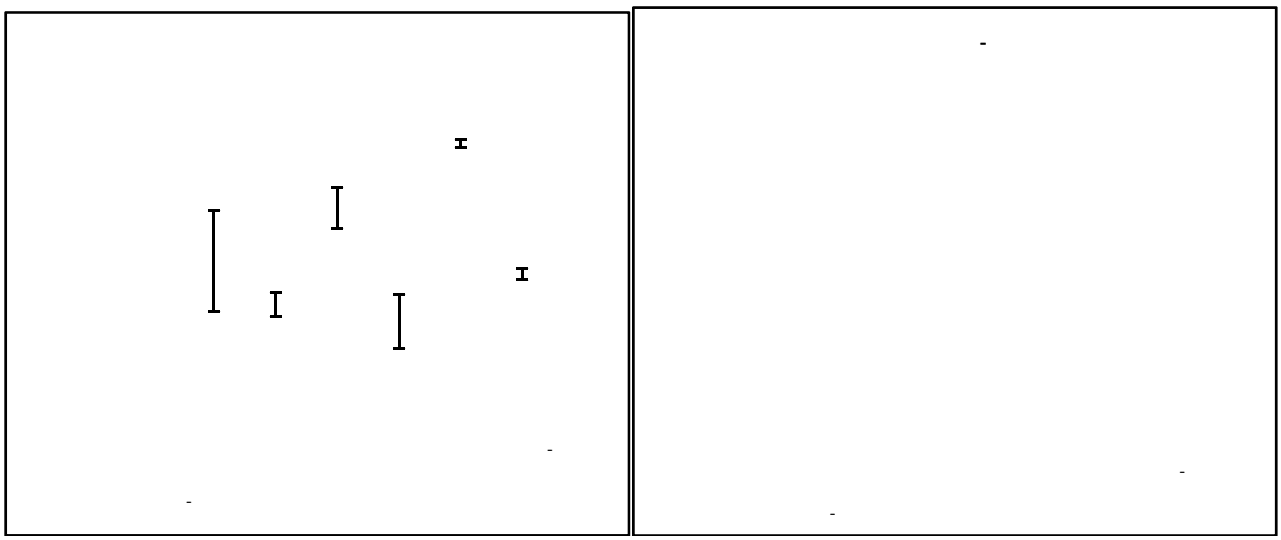


Figure 11: Left panel: Average DNA concentrations across varying elution buffers and a 100ul and 200ul elution volume; Right panel: Total average yield with same parameters.

The results of a comparison of 100 and 200ul elution volumes, where the input sample volume for all studies was 50ul is shown in Figure 11. This figure also compares varying elution buffers and the use of heated buffers. Overall, the non-heated Tris-HCl elution buffer worked as well if not better than the NaOH elution buffer. The heated Tris solution yielded the highest quantities of DNA, however from a processing stand-point, this added procedural step (heating the Tris-HCl solution) may not be necessary if sufficient quantities of DNA are obtained from non-heated options, which was the case. Also, no

measureable differences were noted between the 100ul and 200ul elutions; this indicates that sufficient elution is taking place in the first 100ul eluted in a TruTip™ sample.

R1.3 Sample Preparation Automated Sub-Circuit: The core of the sample preparation subcircuit is our proprietary porous glass matrix. This matrix is compatible with standard chaotropic nucleic acid binding and elution chemistry, but offers these additional advantages: 1) rigid for simple assembly of subcircuits and allowing bidirectional flow for faster extraction and elution kinetics, 2) large porosities for rapid processing of high volumes of viscous samples (e.g. semen), and 3) relatively thick for high binding capacity. In additon, the matrix can be made into almost any geometry allowing tremendous flexibility in subcircuit design.

In this project, we implemented a matrix geometry that positioned the matrix in a 2 ml Rainin pipet tip (Figure 12). The pipet tip form factor (TruTip) allowed testing and optimizing sample preparation protocols for semen and oral swabs using a Rainin electronic pipet. As protocols were established, a sample preparation subcircuit was designed that consisted of a machined cartridge with inlets, outlets, and a receptacle for attaching a TruTip. The TruTip is disposable and replaced after each run. The machined cartridge and other liquid handling components that the sample comes in contact are presently reusable for rapid prototyping and protocol development. However, as the project progresses into to the integrated cartridge in Phase II, all components and lines that the sample comes into contact with will be part of a one-time use disposable - sample will not come into contact with reusable components and lines. The pipet tip form factor will be maintained in the integrated cartridge that contains the sample preparation, PCR, and microarray subcircuits.

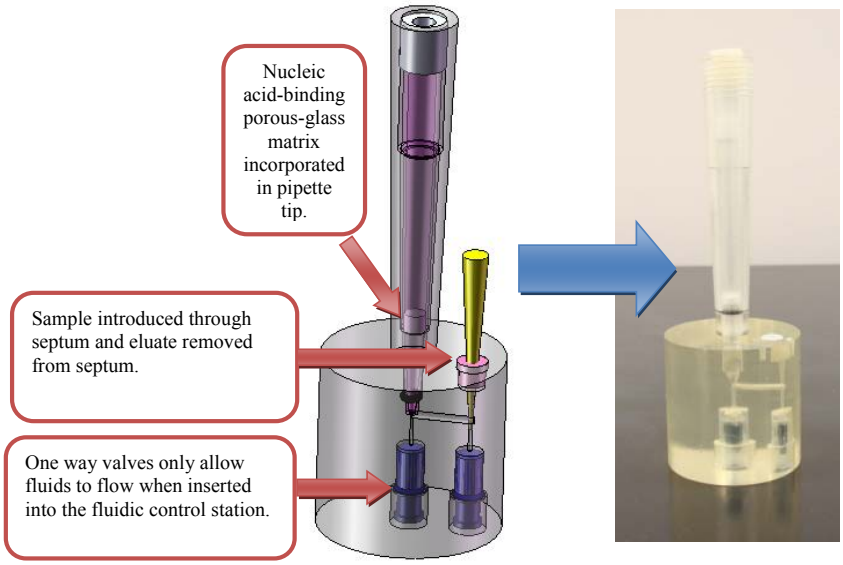


Figure 12: Design and prototype of sample prep cartridge. The sample is introduced through a septum that seals after removal of the tip. A chatrophe is added by a Flow Control Station and mixed on the cartridge. The sample is discarded to waste.

Fluid flow in the cartridge connected to the Flow Control Station as shown in Figure 13. The Flow Contols Station contained a pump, multi-port valves, and the bulk reagents. The Flow Controls Station metered and moved fluids from the bulk reagents to the cartridge. Protocols optimized using the Rainin pipet were converted to scripts on the Flow Contol Station.

Figure 13. Flow Contols Station containing a pump, multi-port valves, bulk reagents and the sample prep subcircuit.

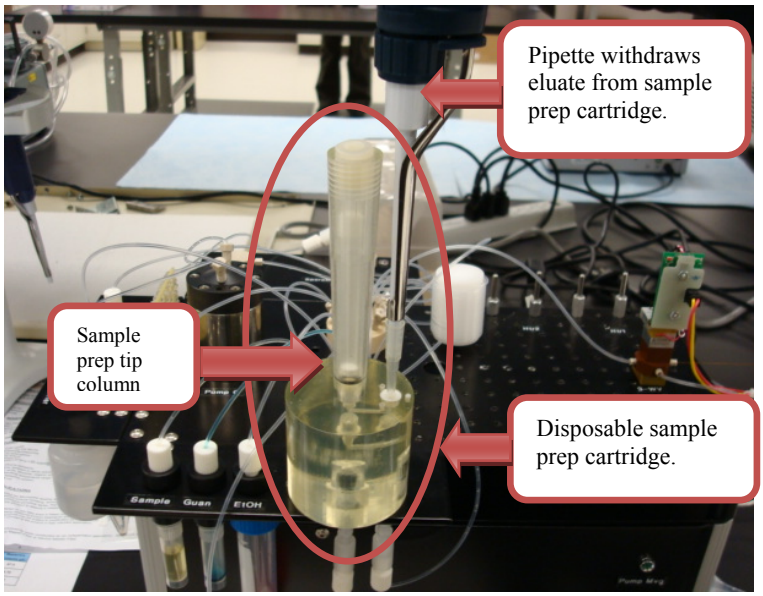


Figure 14. Protoype sample preparation subcircuit to demonstrate automated nucleic acid purification from semen. The subcircuit is connected to the Flow Control Station. Also shown is the pipettor, used to withdraw the eluate following the automated purification process.

A self-sealing entry port for a pipettor (shown in Figure 15) allows easy introduction of the sample without the risk of opening caps, which are often a cause of contamination. Additionally, the one-way valves, shown in Figure 15, make cartridge insertion and removal simple and easy without the risk of losing sample due to leakage after the process is complete.

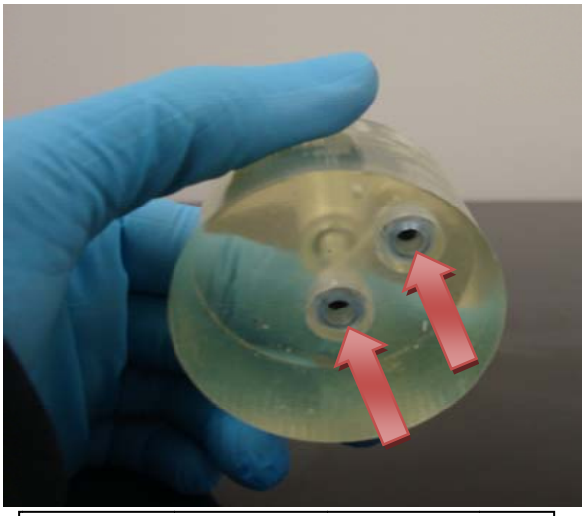


Figure 15. One-way valves on sample prep cartridge allow easy insertion into Flow Control Station and prevent leaking of sample from the cartridge after it's removed.

Three semen samples were sequentially processed on the automated system. In the protocol, the semen sample was added to holding tube that also contains the other lysis components. The mixture was incubated for 30 min, then the automation script was initiated to perform the bind, wash, dry, and elution steps. The system was extensively flushed with water after each run, and some of the water flush after the third run was collected and saved to assess for carryover. Some carryover was expected since we did not implement harsh decontamination procedures since there was uncertainty on how harsh decontamination solutions would react to some of the materials, and the ultimate goal is to confine sample to the disposable.

As shown in Figure 16 (lower panel), the three replicates had better CTs than that of the unprocessed sample, indicating the purification was successful. Carryover contamination was evident as indicated by a signal from the water flush. However, the CT was about 2 cycles higher (similar to the signal obtained from unprocessed) than that obtained from the elutions. Therefore, the carryover was not a significant contributor to the signals obtained from the eluted fractions. When a 10-fold higher sample of semen was used (upper panel), the PCR signal from unprocessed semen was highly inhibited, whereas the processed semen sample displayed a strong positive PCR signal.

As more of the fluid lines are moved to the integrated cartridge to minimize dead volumes, and more dedicated single valves are introduced, the automation process will get more efficient and faster. In addition, we will implement a more stringent decontamination procedure to minimize carryover as we proceed forward with relatively expensive machined parts. No decontamination procedure will be required in the more finished cartridges that will be from inexpensive molded parts for one-time use disposables.

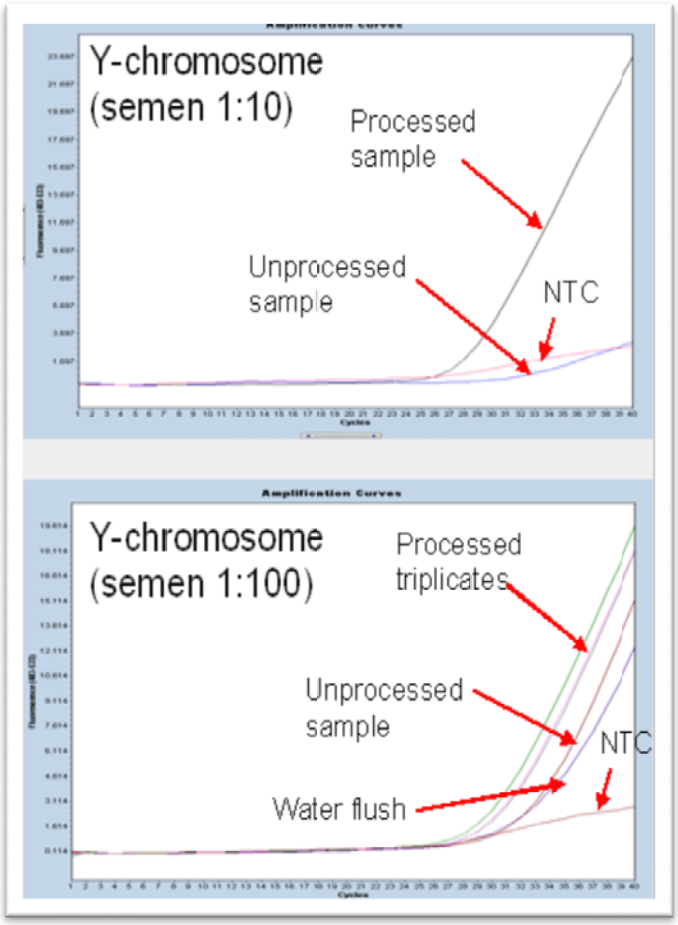


Figure 16: Preliminary results from the sample preparation subcircuit

R2. Hybridization Array with Conventional PCR.

R2.1. Four-plex PCR amplification and detection:

Partial functionality of the assay was tested with four-plex amplification and detection. The primer pairs for M2, M175, M269, and Amel were used. After some preliminary experiments, the preferred concentrations of primers for the multiplex were chosen to be 400 nM for each forward primer and 60 nM for each reverse primer (20:3 ratio). While single-plex amplification showed correct amplification and expected formation of single-stranded amplicons even with lower amounts of reverse primers (40 nM; 20:2 ratio; Figure 17), products of multiplex amplification with 20:3 ratio (Figure 18) gave more stable amplification and higher hybridization signals. While a similar pattern of amplicons were observed for different male DNA samples (Figure 18), control sample F (female DNA only) yielded the expected AmelX PCR result only. Hybridization data (data not shown) further confirmed the formation of correct PCR products. Results of ratiometric analysis of hybridization data are presented in Table 5. All SNPs presented in NIST specification worksheet for SRM 2395 kit were identified correctly. In addition, both amplification and hybridization data show success with using TruTip protocol for isolation human genomic DNA from semen (CEZ sample).

Table 5: Analysis of hybridization data for different samples for the 4-plex amplification. Ratios between signals of probes for different SNPs and presence/absence (+/-) of AmelX and AmelY signals.

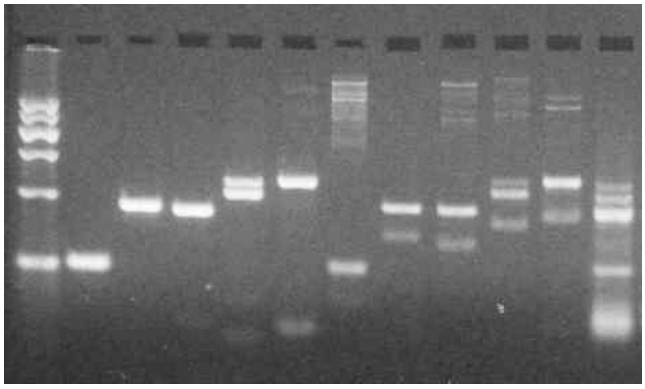


Figure 17: Products of single-plex symmetric (200 nM forward (F) and reverse (R) primer) and asymmetric (400 nM F and 40 nM R) and 4-plex asymmetric (400 nM F and 40 nM R primer) amp for Sample E. Lane 1: 100 bp Marker (Qiagen); Lane 2: M2 primers, symmetric; Lane 3: M175 primers, symmetric; Lane 4: M269 primers, symmetric; Lane 5: Amel primers, symmetric; Lane 6: Experimental Amel primers, symmetric; Lane 7: M2 primers, asymmetric; Lane 8: M175 primers, asymmetric; Lane 9: M269 primers, asymmetric; Lane 10: Amel primers asymmetric; Lane 11: Experimental Amel primers, asymmetric; Lane 12: Four-plex asymmetric**.**

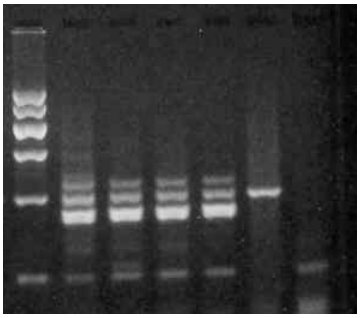


Figure 18: Products of 4-plex asymmetric amplification (400 nM of forward primer and 60 nM of reverse primer; M2, M175, M269, Amel primer pairs) for different samples. Lane 1: 100 bp Marker (Qiagen); Lane 2: Sample A; Lane 3: Sample C; Lane 4: Sample E; Lane 5: Sample CEZ; Lane 6: Sample F; Lane 7: Control (No DNA).

R2.2. Six-plex amplification and detection: Six-plex amplification also worked for the array-based identification of SNPs. For these studies, M2, M170, M175, M269, Chr4, and Amel primers were used. Single-plex PCRs showed specificity of amplification for male DNA sample except M170 amplification (Fig.18). For M170, unexpected formation of a second product was observed. Most likely the formation of the second longer M170 amplicon was associated with non-specific annealing of M170 forward primer to position 173-202 (in addition of correct annealing to 239-258 for rs2032597) despite 3 of 4 mismatches at 3' prime end. The size of this possible second amplicon should be 206 bp, which corresponds to the size of M170 second amplicon observed on the gel. However, the false annealing and the formation of the second PCR product seem to be acceptable since we still observed amplification of the product from the target region. In addition, it should be noted that with the primer concentration ratio used for single-plex PCR (400 nM of each forward primer and 100 nM of each reverse primer; 20:5 ratio), dominant products of the PCRs are double-stranded ampilcons. Since the efficiency of staining of single-stranded short amplicons with ethidium bromide is much less than for double-stranded amplicons, under chosen conditions only double-stranded amplicons are observed on a gel (compare Figures 17 and 19). Single-plex PCRs for the control female sample showed the expected formation of only Chr4 and AmelX amplicons and no amplification with other primer pairs.

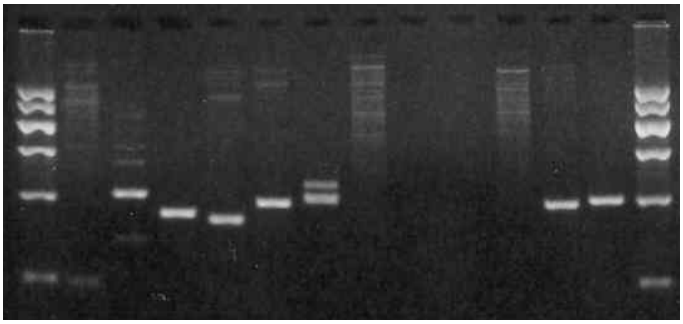


Figure 19: Products of single-plex asymmetric (400 nM of forward primer and 100 nM of reverse primer) amplification for Sample E (lanes 2-7) and Sample F (lanes 8-13) with M2, M170, M175, M269, Chr4, and Amel primers, respectively.

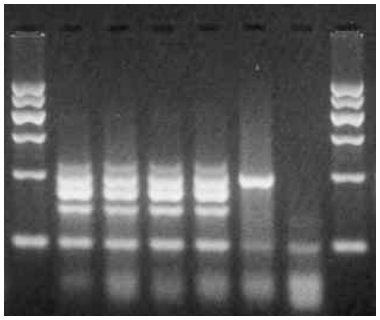


Figure 20: Products of 6-plex asymmetric amplification (400 nM of each forward primer and 100 nM of each reverse primer; M2, M170, M175, M269, Chr4, Amel primer pairs) for different samples. Lanes 1 and 8: 100 bp Marker (Qiagen); Lane 2: Sample A; Lane 3: Sample C; Lane 4: Sample E; Lane 5: Sample CEZ; Lane 6: Sample F; Lane 7: Control (No DNA).

The multiplex amplification product for samples A, C, E, CEZ, and F (Figure 20) showed the expected pattern of amplification. Analysis of fluorescent signals (e.g., see Figure 21) confirmed the presence of all amplicons for all samples. Analysis of ratios between probe signals (Table 6) correctly identified the known SNPs.

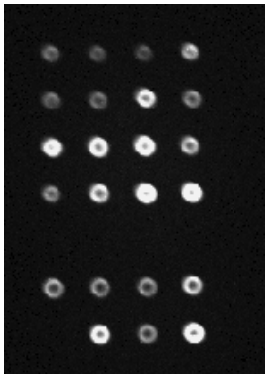


Figure 21: Fluorescent image of TruArray after hybridization with products of 6-plex amplification (Sample E).

Table 6: . Analysis of hybridization data for different samples for the 6-plex amplification. Ratios between signals of probes for different SNPs and presence/absence (+/-) of AmelX and AmelY signals.

	M2_A/ M2_G	M170_A/ M170_C	M175_D/ M175_I	M269_C1/ M269_T1	Chr4_A/ Chr4_T	AmelX	AmelY	Identified SNPs and genes
Sample A	2.03	1.21	0.46	2.21	1.72	+	+	M2_A, M170_A, M175_I, M269_C, Chr4_A, AmelX, AmelY
Sample C	0.41	1.35	0.50	0.60	1.74	+	+	M2_G, M170_A, M175_I, M269_T, Chr4_A, AmelX, AmelY
Sample E	1.80	0.48	0.50	0.68	1.80	+	+	M2_A, M170_C, M175_I, M269_T, Chr4_A, AmelX, AmelY
Sample CEZ	1.49	1.17	0.35	2.91	0.72	+	+	M2_A, M170_A, M175_I, M269_C; Chr4_T, AmelX, AmelY
Sample F	N/A	N/A	N/A	N/A	1.38	+	-	Chr4_A, AmelX
Control (No DNA)	N/A	N/A	N/A	N/A	N/A	-	-	

R3. Allele-Specific Arrayed Primer Extension.

R3.1. Optimization: Various reaction conditions were tested to determine the optimal performance parameters for this assay. For example, multiple polymerases were tested for best incorporation efficiency for Alexa-546-dUTP and best signal to background (data not shown). Deep Vent Exo- exhibited better performance in both of these areas over Vent Exo- DNA polymerase (NEB) and the Stoffel Fragment of Taq polymerase (ABI). Perfect Match solution (Stratagene) which is marketed to improve the specificity of Taq polymerase was tested and found to have little to no effect on Deep Vent Exo- polymerase as far as improving the signal difference between the two allele specific primers (data not shown). Also, adding in the forward primer in solution increased the signal in most cases, but the simplicity of linear amplification was determined to be optimal for this application. The dNTP concentrations were varied at different ratios of labeled-dUTP to unlabeled dNTPs with the optimal ration of 1:10 (1uM labeled to 10uM unlabeled).

Using the optimized method described above in section M3, Figure 22 shows the resulting ratio of fluorescence signals from one AS-primer to the other. For example, for M2, A and G are the two possibilities for this SNP site. The ratio of A/G and G/A indicates which allele is present. A ratio greater than 2 indicates the SNP allele in the numerator is the one present in the sample. As shown in Figure 22, AS-APEX analysis of samples C, E and 3 yields the correct genotype with all AS-ratios greater than three and some as high as nine.

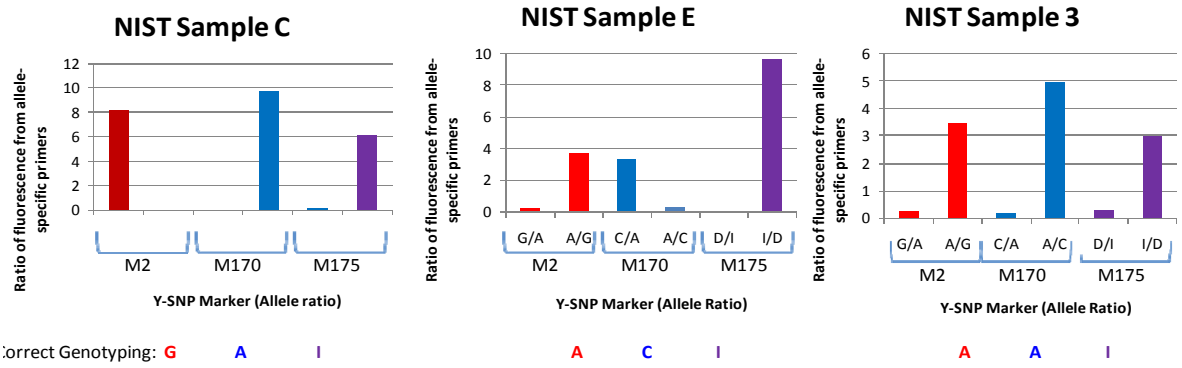


Figure 22: Allele specific primer ratios for multiplexed AP-APEX assays for three NIST samples with correct genotyping results.

Further optimization for the M175 APEX primers was required because the insertion primer was found to be favored for targets with both insertions and deletions. One iteration of primer redesign that shifted the primers by a few bases yielded the results shown in Figure 23. NIST samples C, 1, and 2 are typed correctly using the M175 AS-APEX assay.

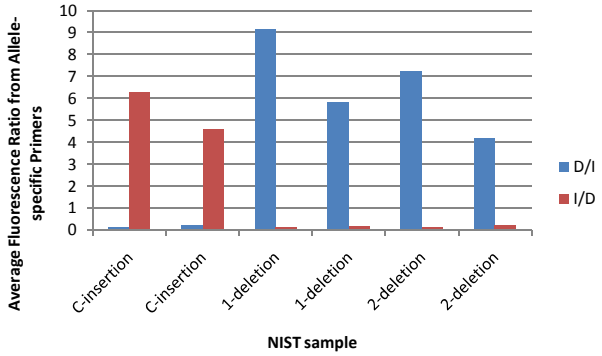


Figure 23: Allele specific primer ratios for multiplexed AP-APEX assays for three NIST samples with correct genotyping results for the M175 allele. D= deletion, I = insertion.

Background fluorescence was observed in the negative controls at both 100 and 200uM, but the 10µM negative control was consistently clean, so this was the concentration used for all data analysis. Furthermore, several primers of different lengths were designed for each SNP allele as previously mentioned and listed in Table 4. The shortest primers (with lowest melting temperatures) were shown to give the best discrimination between the two alleles. These results correspond to the hypothesis that a mismatch will have a greater effect on the melting temperature of a shorter oligo compared to a longer oligo, and thus, lead to better discrimination. The optimal immobilized AS-primers were re-printed on a down-selected array v2.1 for an "easy-call" array whereby visual analysis lends to straightforward interpretation. Figure 24 shows the down-selected array images for several Y-chromosome DNA samples. The correct genotype is visually apparent for each sample.

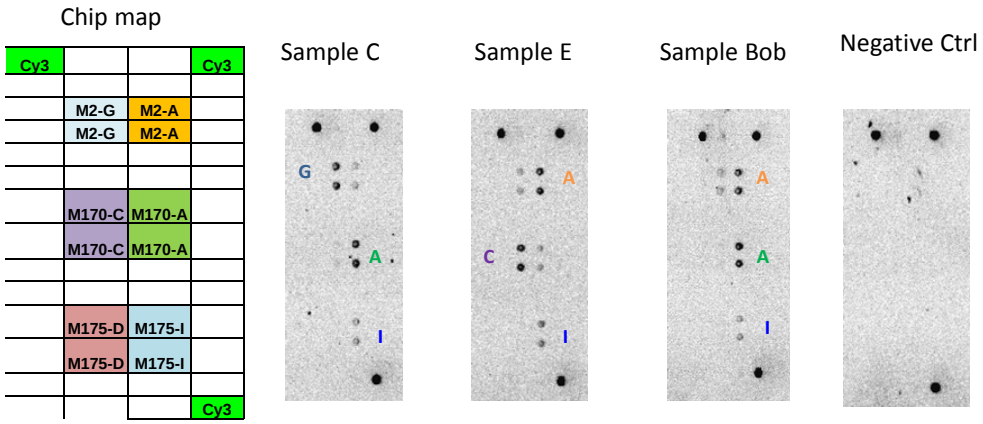


Figure 24: Down-selected arrays for visual analysis of multiplexed AS-APEX assays on Sample C, E, "Bob" and negative control. The chip map on the left indicates the assay (M2, M170, or M175) and the 3′base of the allele-specific primer (G, A, or C). D and I denote deletion (or lack of insertion) and insertion, respectively. Sample "Bob" is a semen sample with the genotype A, A, I for M2, M170, and M175 respectively.

R3.2. Sensitivity Study: We tested the limit of detection of the AS-APEX assay using a standard sensitivity curve with neat male DNA. We PCR amplified 1ng, 100pg, and 10pg of male DNA using the 3-plex PCR primer set in triplicate. A gel of the reaction products showed missing product bands for the M2 and M170 products in the 10pg reactions (data not shown); so the APEX assay was run on the 1ng and 100pg amplified products. Figure 25 shows the correct typing results for both APEX reactions, demonstrating sensitivity down to 100pg or roughly 16 genomic copies.

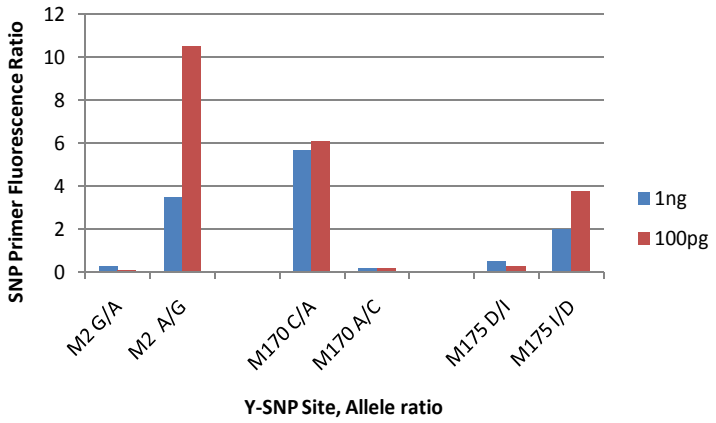


Figure 25: Sensitivity study results. Allele specific primer ratios for PCR amplified NIST samples starting with 1ng (blue) or 100pg (red) genomic DNA.

We also tested male DNA samples spiked with female DNA at various concentrations.
For this mixture sensitivity study, ratios of male:female DNA were the following: 0:1, 1:0, 1:1, 1:2, 1:5, 1:10, 1:25, 1:50, 1:100 respectively, keeping the male DNA constant at 100pg (except for the 100pg female-only sample). Product gels of the PCR amplified products of these mixtures showed all three bands present in triplicate with the same intensity regardless of the varying amount of female DNA present (data not shown). The amplification of 100pg female DNA resulted in no product bands on the gel and no fluorescence signal from the APEX assay (data not shown). Figure 26 shows the fluorescence

ratio APEX results of the highest stringency conditions at 100:1 female:male (10ng and 100pg respectively).

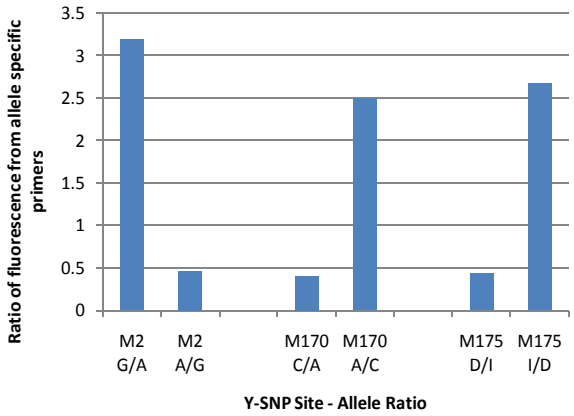


Figure 26: Allele specific primer ratios for APEX assay using PCR product from 10ng female and 100pg male DNA. Results are an average of four replicates.

R3.3. Sample-to-Answer Demonstration: A semen sample was processed through the TruTip extraction procedure, then amplified by PCR either in a single-plex assay on the bladder thermocycler or in a multiplex on the MJ thermocycler. Resulting amplicons were added to the APEX assay for final SNP discrimination. The results shown in Figure 27 demonstrate significant discrimination between the different AS-APEX signals and yield the correct genotype for the end-to-end processed sample. These samples have also been run in a modified flow cell with similar results (data not shown).

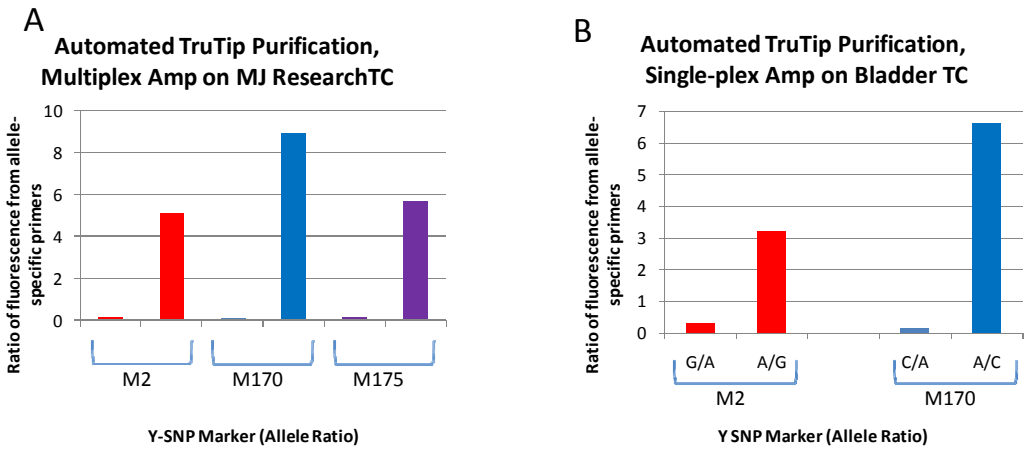


Figure 27: Allele specific primer ratios for multiplexed (A) or single-plex (B) AP-APEX assays for three NIST samples with correct genotyping results.

R4. PCR and Microarray Sub-Circuit.

Various iterations of subcircuits based on the two approaches shown in Figure 28 were designed and tested. The first type of subcircuit uses a single chamber to perform both PCR and microarray hybridization. The chamber contains a gel-spot microarray and also serves as the reaction chamber for thermal cycling. PCR reaction mix containing template is loaded into the chamber, thermal cycling is performed, and the product hybridizes to its respective immobilized probe(s). The second and more traditional approach utilizes two chambers, one for PCR and the other for microarray hybridization. In both approaches the reaction chamber is rigid to accommodate printing the gel-spot array.

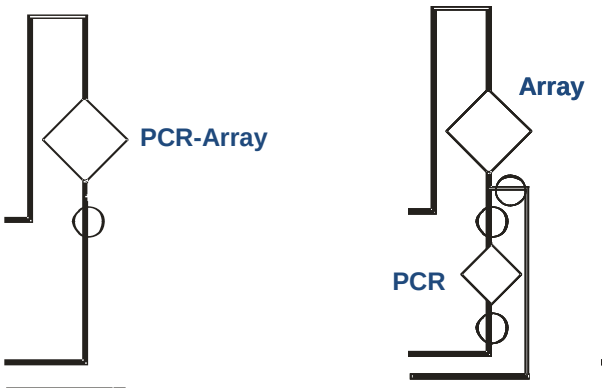


Figure 28. Approaches for integrating PCR and microarray hybridization on a subcircuit. (Left) PCR and microarray hybridization occur simultaneously in the same chamber. (Right) PCR and microarray hybridization performed sequentially in separate chambers. Circles denote valve positions.

The bladder thermal cycler as shown in Figure 29 was developed as a preferred means to interface thermal cycler heaters with the two types of subcircuits. The bladder thermal cycler alternatively gates two temperature-controlled fluids through a pair of bladders that tightly press against the opposite walls of the flat reaction chamber of the subcircuit.

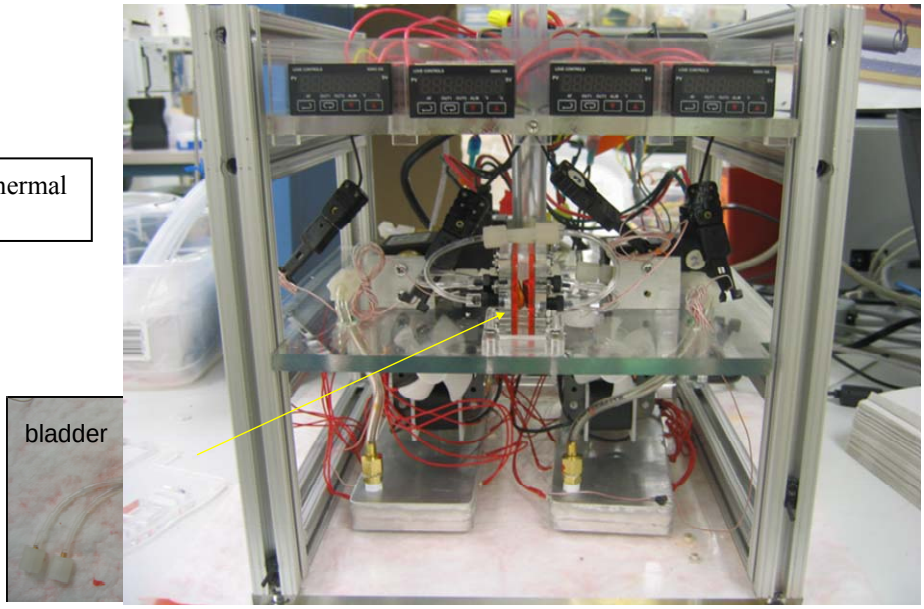


Figure 29. Photograph of the bladder thermal cycler.

A fluid flow diagram illustrating the concept is shown in Figure 30. A dual-loop circulation system maintains two temperature zones of circulating fluids. Each zone has a dedicated heat exchanger and pump. Three 3-way valves are used to divert the desired temperature fluid through the bladder pair. Re-circulating fluids are heated through in-line heat exchangers, thereby reducing the volume of heat transfer fluid to approximately 100 ml, yet maintaining a steady-state set temperature in each temperature zone. If a third temperature step is required, an additional loop can be added or controlled mixing of the dual loops could be considered.

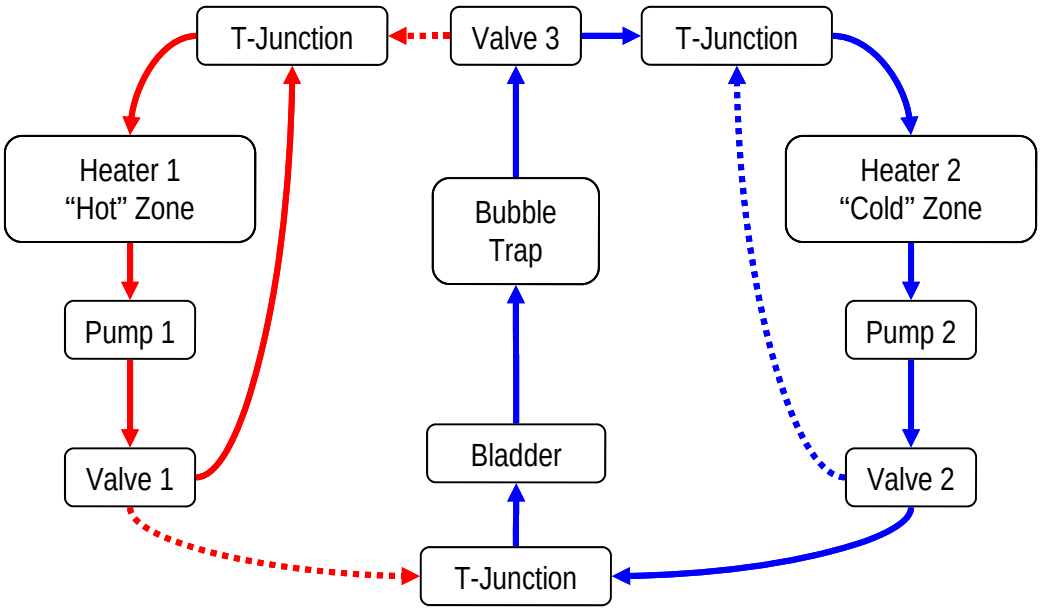


Figure 30. Flow diagram of the dual-loop circulation system in the bladder thermal cycler. Depicted is fluid from the cold zone (blue) being directed through the bladder for the annealing step of PCR while the hot zone (red) re-circulates only within its loop. For the denature step, the hot zone will be directed through the bladder and the cold zone will re-circulate only within its loop.

Figure 31 shows temperature profiles of a thermal cycling run performed with the dual-loop bladder thermal cycler with set points for 94°C for 1 sec to denature and 65°C for 25 sec to anneal and extend. Temperatures inside the first heat exchange block (i.e., the hot zone), the second heat exchange block (i.e., the cold zone), and the reaction chamber were measured during the course of the run. The hot zone and cold zone temperatures remained at steady-state. The zones are held at a slightly elevated or reduced temperatures (101°C for the hot zone and 63°C for the cold zone) than the target temperature of the thermal cycling protocol to create a temperature offset that compensates for heat losses in the fluid paths from the temperature zones to the bladder assembly and heat transfer from the hot zone to the cold zone. The hot zone temperature offset can be minimized by reducing the path length that the circulating temperature control fluid has to travel and by utilizing insulation materials around the fluid paths. The successful thermal cycling temperature profile, albeit not perfect, demonstrated that the principle of switching temperature control fluid through the bladder assembly can deliver the intended control of temperature in the reaction chamber. The ramp down to 65°C experienced a decrease in the ramp rate at 66.5°C due to effects of some mixing of hot zone fluid in the cold zone loop during the initiation of the cold zone fluid through the bladders. Some fixes to this observation include replacing the fixed speed

pumps with variable speed pumps. Variable speed pumps should permit fine tuning of temperature control, for example, by utilizing a feedback loop between the temperature sensors and the pump speed control. In the case of the 65°C step, a feedback loop will be established to increase the pump speed of the cold zone pump as the bladder approaches 66.5°C. Higher turnover of the mixed heat transfer fluid should increase the rate at which this fluid reaches steady-state.

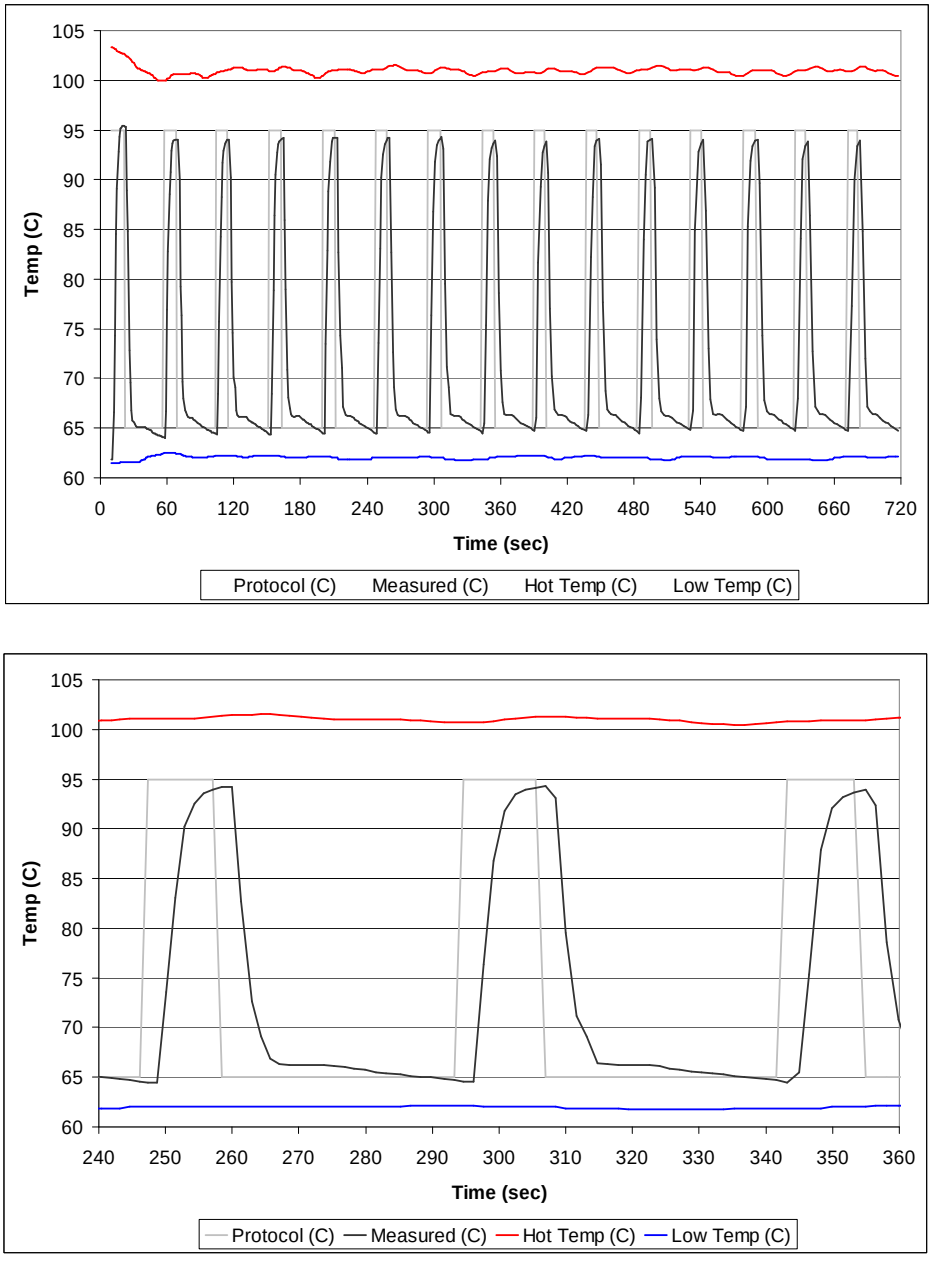
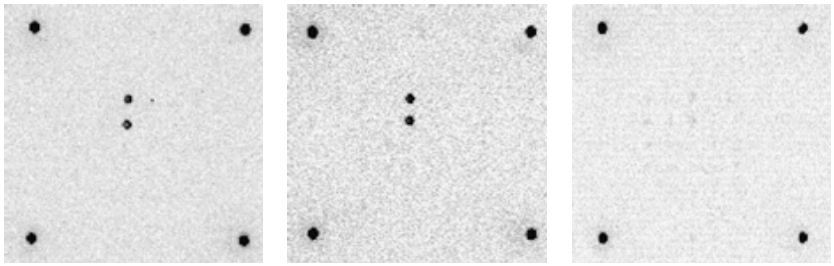


Figure 31. Temperature profiles generated from the bladder thermal cycler. The heating and cooling rates averaged 13.6°C and 17°C, respectively. PCR in a flow cell was accomplished by performing 40 cycles of thermal cycling in about 40 minutes.

Conventional thermal cyclers that perform heating and cooling at a fixed site achieve faster thermal cycling by inducing brief overshoots and undershoots of the heat exchanger temperatures relative to the setpoint and reaction chamber temperatures. Sophisticated software algorithms that tightly control and coordinate the heating (e.g., resistive, peltier) and cooling (e.g., thermoelectric, peltier, refrigerant, fan, etc.) components are necessary to ensure the reaction temperature reaches and plateaus at the setpoint temperature. Others have simplified temperature control on thermal cyclers by moving a PCR reaction among two or more sites at fixed temperatures [32-35]. Our dual-loop circulation in conjunction with the bladder heaters permits simplified temperature control while keeping the reaction stationary at a single site.

As a demonstration of implementing the bladder thermal cycler for the single chamber approach to perform coupled PCR and microarray hybridization, our well characterized biothreat microarray (Figure 32) was used for the study. 1000 copies of *Bacillus anthracis* genomic DNA was mixed with PCR master mix and loaded into the PCR/microarray subcircuit. The subcircuit was postioned on the bladder thermal cycler and subjected to coupled thermal cycling and hybridization. The resultant microarray reader images from the *B. anthracis* DNA samples displayed positive signals only at the correct *B. anthracis* gel-drop probes. In addition, no positive signals were evident in coupled PCR and hybridization test using no target (negative control).

Cy3									Cy3
			E	**E**	**E**	**E**			
		NSN	Yp-1	Ba-3	Vac-1	VEE-1	NSN		
		NSN	Yp-1	Ba-3	Vac-1	VEE-1	NSN		
		NSN	Yp-6	Ba-6	Vac-4	VEE-1	NSN		
		NSN	Yp-6	Ba-6	Vac-4	VEE-1	NSN		
			E	**E**	**E**	**E**			
Cy3									Cy3



10^3 copies Ba 10^3 copies Ba negative control

Figure 32. Microarray results from coupled PCR amplification and hybridization of *B. anthracis* (Ba) genomic DNA performed using the flow cell and the bladder thermal cycler. (Top) Microarray map of gel spots in the flow cell reaction chamber. Cy3 = Cy3 fluorescent dye position markers, NSN = nonsense probes, E = gel elements with no probes, Yp-1 = *Y. pestis* probe 1, Yp-6 = *Y. pestis* probe 6, Ba-3 = *B. anthracis* probe 3, Ba-6 = *B. anthracis* probe 6, Vac-1 = Vaccinia virus probe 1, Vac-4 = Vaccinia virus probe 4, VEE-1 = Venezuelan equine encephalitis probe 1. (Bottom) Reverse fluorescent images of two flow cells that had 10^3 copies of *B. anthracis* genomic DNA target in the reaction and one flow cell that contained no target (negative control) in the reaction. Positive signals (Cy3 markers and Ba-3 probes) correspond to shaded areas in the microarray map. The Ba-6 probes were designed for a different amplicon and remained negative.

Conclusions

Discussion of Findings

The goal of this Phase I project was to demonstrate the feasibility Akonni's sample preparation and microfluidic microarray subcomponents for analyzing SNP markers (e.g., Y-SNPs) using forensic-type samples. In this study, we were able to demonstrate successful in-cartridge DNA extraction from semen and oral (saliva) samples, successful multiplexed PCR amplification without the need for sample splitting (with various biochemistries and sub-circuit/array configurations), and successful DNA analysis using a well-established microarray platform with forensic-type samples.

As described in this report, two sub-circuits were developed and tested: 1) the sample preparation sub-circuit and 2) the PCR/array sub-circuit. Studies for sample preparation included use of the TruTip as a stand-alone method for extraction and the incorporation of the TruTip form factor into an integrated Flow Control System. We observed that the TruTip™ Easy protocol is more than sufficient to yield adequate quantities of human DNA from saliva (over 1ug amounts) which would be suitable for forensic analysis. This protocol does not require protease enzymes or heat in contrast to commercially available kits (e.g., Qiagen's DNA mini kit). It is extremely fast with a sample processing time of approximately 4 minutes or less and a minimal starting volume of liquid saliva (50ul). Overall, liquid saliva proved to be an easy sample type for collecting, storing and processing and therefore, easily amenable to forensic applications. Success using the automated microfluidic flow control system for sample preparation using semen samples as the starting material further demonstrates the feasibility of incorporating sample preparation sub-circuits within an end-to-end push button technology suitable for forensic applications. Follow-on studies will leverage off of these initial studies regarding testing additional sample matrixes (e.g., blood, semen, swabs, etc.) along with expanded sensitivity studies.

Multiplex asymmetric amplification with hybridization on TruArray containing probes targeted to different Y-chromosomal and autosomal SNPs showed applicability for correct identification of the SNPs of interest. The experiments for the multiplex PCR optimization, optimization of probe design (in order to get higher signals for some SNP probe pairs and balanced hybridization pattern), optimization of hybridization parameters, and sequencing of the regions of interest for uncharacterized samples can be explored for further utility. In addition, successful end-to-end processing of a forensic type sample was demonstrated using the TruTip sample extraction protocol for isolation human genomic DNA from semen (CEZ sample; Table 5) followed by TruArray analysis using conventional PCR and hybridization. Although we succeeded in correctly genotyping the samples tested, the signal ratios and margin of error was lower compared to the AS-APEX assay. Therefore, APEX was the chosen method moving forward. During the testing process our assay revealed the correct typing of NIST sample 3, which had been incorrectly labeled by NIST as a deletion instead of an insertion for the M175 site. NIST verified that our result was correct and were pleased that we had brought the error to their attention. We have shown that the assay is highly sensitive with detection limits at 16 copies of genomic DNA. With further optimization we believe the assay could achieve the same single-copy detection capability as regular PCR. We optimized our reaction conditions and primer sequences to be highly selective with the ability to discriminate between single nucleotides in a high background of female DNA, a situation highly relevant in rape cases. The ratio range tested (0-10ng) was a result of material constraints of the available NIST samples. In future studies a wider range could be tested with higher concentration

samples. Further studies could include increasing the ratio mixtures with higher concentrations of female DNA as well as testing 10ng or higher amounts of female-only DNA as a control.

Because the AS-APEX assay is a more specific reaction compared to hybridization, our typical background signal is very low. We designed the immobilized primers employed in AS-APEX extension to be located in the middle of the target amplicon with no homology to PCR amplification primers. Furthermore, we ran the assays under very stringent conditions, including low salt, low dNTP/labeled-dUTP concentrations and high temperature. These conditions, along with the primer design, allow for single nucleotide discrimination in which the 3' base dictates the extension efficiency even with the full complementarity of the remaining primer bases. Therefore, even if primer-dimers or other amplification artifacts are created during the PCR step, these by-products will not lead to primer extension and subsequent label incorporation with the level of specificity gained by the AS-APEX assay. We have observed high background fluorescence from immobilized primers that have 3' self-complementarity. Similar to solution phase PCR, this fluorescence can be explained by homo-dimer formation due to the interaction between immobilized primers within the same gel element in the absence of target. These primers simply require a redesign to eliminate the complementary sequence and eliminate background.

Further optimization of our AS-APEX assay system will involve steps to further increase the SNP discrimination as well as to simplify the assay and minimize the reaction time. For example, we plan to examine different primer sequence modifications to increase the overall APEX signal and discrimination. We will also test approaches to eliminate the PCR amplification step by using whole genome amplification or combining the amplification reaction with the APEX assay for an "all-in-one" system. Both approaches would be tested in a flow-cell design, which allows for reduced contamination within a sealed, thermal stable reaction chamber in contrast to the BioRad frame seal chambers which require opening to the environment for washing, drying and imaging. The eventual goal is to amplify the purified sample on the Bladder Thermal Cycler (in a multiplex assay), and perform the AS-APEX assay in a flow cell on the Bladder as well. Ultimately, each of these components will be integrated into one cartridge with a flow path between each step for a fully automated sample-to-answer system.

The fluidic control of the bladder has been demonstrated to be very robust in our prototype system. To address any concerns about complicated fluid controls, it should be noted that similar approaches that do not involve thermal cycling have been used to cool computer processors and RAM. These computer coolers are commercially available, and contain pumps and valves to control flow rate and coolant temperature. Customers are willing to circulate fluid within their computer to gain faster processing speed. The fluidic control in the bladder thermal cycler is very basic, only consisting of two pumps and three valves. This novel approach is required to solve the issues with conventional thermal cyclers since they do not provide uniform, fast, and repeatable heat transfer to a rigid, flat PCR chamber. In addition, the bladders substantially simplify the mechanics required (typically involving some type of compression forces) to interface rigid heaters to a rigid flat, wall of a PCR chamber in an integrated, disposable cartridge.

For our standard hydrogel compositions, pore size constraints on target diffusion into the gel element become limiting at target lengths approaching ~500 nucelotides (nt). For this reason, on-chip amplification strategies are designed to target regions < 200 nt in length. In the event that target nucleic acids exceed 500-1,000 nt, then chain extension from a gel element is primarily a surface phenomenon. In this case, the 3-D shape of the gel element still has a competitive advantage over a competing 2D

surface, in that all probes are still sterically accessible and there is more effective surface area within which to perform the chain extension reaction.

In the event that assay sensitivity needs to be improved and/or the process modified to accommodate large, unfragmented DNA targets, then polymers with a range of average pore size (1-1000 nm) and physico-chemical properties (transparency, hydrophilicity, density, etc.) can be synthesized by changing the nature and concentration of polymer monomers, porogen, radical polymerization initiator, and reaction temperature. We have synthesized a range of these polymers and tested their behavior under non-equilibrium hybridization conditions with synthetic DNA targets, but have not yet tested them under an APEX or chain-extension assay other than to show that they survive 100 cycles of thermal cycling without damage or detachment of the gel elements from the substrate. Alternatively, target DNA could be sheared by bead beating or sonic lysis to an average size < 500 nt as part of the sample preparation procedure. These new polymer compositions and approaches could be an object of a Phase 2 series of experiments, if necessary.

Implications for Policy and Practice

Akonni's general business plan and dissemination strategy (for all products) is to involve the expert users in the initial development, design and verification testing of the microfluidic cartridges and products. This global product development plan serves two fundamental purposes for technology dissemination, production and sales: 1) education, user buy-in and involvement in assay format, use, and proper interpretation; and 2) pre-production technology/hardware placement within the user community. Our hope is to continue collaborations with agencies and individuals such as the Massachusetts State Police, NIST and Dr. Eric Buel as these types of relationships help ensure that Akonni is properly addressing standards, controls, and production QA/QC criteria that must withstand scrutiny in a court of law.

As indicated above, instruments will be subjected to extensive marketing and business development input to design and build a commercial alpha unit that 1) is based on user specifications and requirements, 2) consists of components that are subjected to rigorous QA/QC (conforming to ISO 17205) and 3) can be manufactured and assembled in moderate to high volume at a relatively low cost. Ultimately, standard reference materials used for verification and validation testing will be obtained and tested on the prototype device(s) to meet the mandatory requirements set forth by relevant forensic decision making authorities (e.g., the American Society of Crime Laboratory Directors-Laboratory Accreditation Board (ASCLD/LAB), FBI's Quality Assurance Standards). In the course of this process, prototype instruments will be provided to 2-3 designated crime laboratories, with the resulting data providing an initial baseline level of accuracy, precision and reproducibility. This information, along with customer feedback, will be compiled and utilized for alpha unit design and production. The main validation testing will occur during beta unit production in which 10-15 crime labs will be provided instruments. In parallel, Akonni Biosystems will establish either internally or in conjunction with a strategic partner, a commercial production line and begin marketing/selling tests directly to the end users (e.g., state crime labs; familial-testing service providers) or through a product supply agreement with an established forensics vendor (e.g., ABI, Promega).

Implications for Further Research:

The next phases of this effort will build on the successful Phase I component and assay development tasks. Phase II will focus on defining protocols and assay chemistries, and packaging components and reagents into an integrated system for automated, sample-to-answer results. The system will consist of the instrument (i.e., liquid handling, Akonni Bladder Thermal Cycler, Akonni Reader, and cartridge docking station) and a disposable, integrated cartridge (i.e., Akonni TruTip, Akonni PCR and TruArray flow cell chambers, microfluidic circuits, and microfluidic valves). Emphasis will be placed on refining fluid paths (e.g., minimize the number of paths and path lengths), liquid handling and fluidic control (e.g. pumps, valve types and configurations), molding of the disposable plastic cartridge parts, developing an intuitive software and graphical user interface, and reducing the instrument footprint (approximately 2-3 cubic ft). Protocols and assays will be streamlined for minimum complexity, time, and cost. In addition, assay reagents (e.g., PCR, APEX) will be transitioned to a lyophilized format for long term storage and field deployment.

The successful development of an end-to-end SNP-typing cartridge would have direct (technical) implications for translating forensically important assays into a low-cost, user-friendly format. Given the fluidic process described herein, the same basic microfluidic format and manufacturing infrastructure could be applied directly to forensic serology with antigen/antibody testing; mRNA expression studies to determine tissue sources in biological material left at crime scenes; mitochondrial testing; ethnicity or phenotype determinations of semen donors in no-suspect rape cases; plant and/or non-human animal (i.e., dog or cat) genotype analysis; and many others. Given the portability of the TruArray™ system, its low cost and ease of use, the products of this research may also be translated to mobile crime units. This basic test system could aid in stain identification (to determine if multiple stains came from the same person or from different individuals), which could expedite the sample collection process at crime scenes. Finally, applications to forensic biodefense (e.g., anthrax or small pox testing) are today becoming a new and emerging application area that could benefit greatly from an integrated sample-to-answer rapid test platform.

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DISSEMINATION OF FINDINGS

No journal publications to date. Work supported by this grant was presented at the NIJ Conference held in Arlington, VA in July of 2008 by Dr. Philip Belgrader:

Belgrader P, Cooney C, Holmberg R, Knickerbocker C, Kukhtin A, Mokhiber R, Stewart C, Thakore N, Zimmerman C, and Reynolds JE (2008) *A Low-Cost Microfluidic Microarray System for Typing Y-Chromosome SNPs*. The NIJ Conference, Arlington, VA.