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NIJ Final Technical Report

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Abstract:

The purpose and scope of this project was to design, build, and test an integrated microfluidic controlled microarray platform to type SNPs. For Phase II, the SNP application focused on deducing an individual's physical appearance from DNA evidence, with SNPs for eye color determination selected as the model. In Phase I, we demonstrated feasibility of Akonni's gel drop microarray workflow as a complete solution for forensic SNP-typing applications. Sample preparation, PCR, and the microarray were packaged into prototype flow-through microfluidic modules for SNP discrimination. Phase II focused on refining protocols and assay chemistries, and packaging components and reagents into an integrated system for automated, sample-to-answer results. The system consisted 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 was placed on refining fluid paths (*e.g.*, minimize the number of paths and path lengths), liquid handling and fluidic control (*e.g.*, pumps and valve types and configurations), iterative enhancement of the disposable plastic cartridge parts, software and graphical user interface, assay stabilization, and instrument footprint (approximately 2-3 cu. ft). Performance criteria for this feasibility study included the capability demonstrating: 1) a multiplex PCR

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and isothermal Allele-specific Arrayed-Primer Extension (AS-APEX) test for eye color, 2) positive results from blood samples processed on the integrated system, 3) positive results on the integrated system using lyophilized assay reagents, and 4) correct SNP typing of mock forensic samples on the integrated system. The major deliverable for this phase is a final technical report supporting substantial progress on these tasks.

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EXECUTIVE SUMMARY

Akonni Biosystems, Inc. was awarded a grant from the National Institute of Justice, Office of Justice Programs, in October 2009 to integrate Akonni's patented sample preparation (TruTip™), thermocyling, and gel element microarray (TruArray™) technologies for the typing of SNPs for forensic applications. Eye color SNPs were chosen as the model system for this demonstration. Over the last few years, there has been a tremendous understanding of the linkage of human physical appearance to SNP genotypes. While there can be many SNPs associated with a specific aspect of physical appearance, such as eye color, the SNPs can be down selected to only a few key genetic predictors. For example, six SNPs have been determined as the major genetic markers for eye color, and hair color can be also associated with some of these SNPs.

The goal of the Akonni integrated system is to incorporate state of the art microfluidic technology to automate processing from sample preparation to allele discrimination, generating key phenoytpe information to aid in rapidly apprehending a perpetrator or identifying a victim. In forensics, this technology could afford users with several key advantages that include:

- rapid processing (sample to answer goal of 2 hours or less)
- walk-away push-button operation (including the sample preparation)
- low-cost (targeting $25K instrument and $10-15 per test)
- self-contained disposable cartridges (substantially reduced chances of cross-contamination)
- small-footprint (targeting 2 cu. ft for an instrument that processes up to 8 samples)
- portability (field or mobile lab use)
- flexible platform (amenable to both nucleic acid and protein targets).

Akonni Biosystems has an exclusive license 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.

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

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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 II of this study, we proposed to demonstrate integrated nucleic acid sample preparation from oral and/or blood samples with a multiplex PCR/AS-APEX test for six eye color SNP markers. Future goals would pursue expanding the SNP panel for physical traits and advancing the hardware design to the alpha unit stage. The specific technical objectives and milestones for the Phase II project were:

1. Refine allele-specific arrayed primer extension (AS-APEX) protocols and chemistries;
2. Design and assemble a system that integrates sample preparation and microarray subciruits;
3. Upgrade the integrated cartridge with lyophilized reagents; and
4. Sample-to-answer testing on the integrated system.

Refine allele-specific arrayed primer extension (AS-APEX) protocols and chemistries: The normal convention to practice AS-APEX 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 product of asymmetric PCR applied directly to the array surface for incorporation of labeled-dUTP and labeled-dCTP 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 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 fluorescently-labeled nucleotides to produce the final signal. We have applied this strategy to the discrimination of eye color SNPs.

In this study, six SNP regions were targeted to deduce eye color. These SNPs have been reported in the literature to be the major genetic markers for eye color, with an overall AUC (integral of the receiver operating characteristic curves) of 0.93 for brown, 0.91 for blue, and 0.72 for intermediate colored eyes. Since there is substantial sequence information available for these six SNPs, they were chosen as the model assay for Phase II. Transforming the sequence information into a functional multiplex PCR-AS-APEX required adjusting primer extension lengths to get sufficient signals from all immobilized, extended primers. Successful demonstration of a multiplex assay to discriminate the six SNPs was achieved. Future work will expand the SNP panel.

Another accomplished goal was transitioning the AS-APEX assay temperature requirements from a cycling program used in Phase I to an isothermal format. This permitted simplification of the instrumentation required to run the assay using our two-chamber flow cell subcircuit on the cartridge. Results have demonstrated the ability to run the AS-APEX assay under isothermal conditions while maintaining discriminating power for all 6 SNPs using 8 ng of DNA. The total PCR/AS-APEX assay

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time was 2.5 hrs which is close to our target time of 2 hrs. Additional optimization and testing is required to make the assay more robust and sensitive.

Design and assemble a system that integrates sample preparation and microarray subciruits: A microfluidic cartridge and companion instrument was developed in this project to support purification of blood using Akonni's TruTip, PCR in a flow cell that is inserted in an Akonni-patented bladder thermal cycler, and hybridization to a microarray that is also in a flow cell. Liquids were introduced onto the cartridge, but did not flow out of the cartridge. Precise liquid control was handled by microfluidic pumps in the instrument and Akonni-designed injection-molded pin valves in the cartridge. Additional cartridge valves required that the cartridge be engaged with the instrument manifold for liquids to flow, thus all liquids remain contained on the cartridge following completion of the run. A number of engineering challenges were encountered, but Phase II culminated in a prototype integrated system consisting of a cartridge inserted into an instrument to perform automated sample-to-answer results. The system architecture remained flexible to allow design and configuration of a commercial-grade system to process multiple samples in parallel in Phase III.

Upgrade the integrated cartridge with lyophilized reagents: Lyophilized pellets for PCR and APEX were produced at Biolyph (http://www.biolyph.com/) according to formulations developed at Akonni. In the first round of lyophilization tests, three sets of PCR and APEX lyophilized pellets (Lyospheres), which included all reaction components, were prepared using different excipient recipes. Lyospheres were tested for singleplex and multiplex PCR and APEX. In the next round of tests, enzymes and buffers were lyophilized independently to obtain a better understanding of the effects of lyophilization on reaction components. Each of lyophilized components was tested in combination with each other or wet chemistry (*i.e.*, lyophilized PCR buffer was combined with fresh Taq Polymerase or vice versa). Positive results were obtained using the Lyospheres, with the APEX Lyospheres displaying better performance than the PCR Lyospheres. However, studies in which Taq polymerases and PCR buffers were lyophilized independently revealed that the enzymes remained stable but the buffers became problematic. Future work will involve improving PCR buffer stabilization during lyophilization, or as an option, keep the buffer as wet chemistry since use of lyophilization is primarily to maintain enzyme stability.

As a result of the extensive time and resources in both Lyosphere and integrated system development, we were not able to utilize the Lyospheres for sample-to-answer system testing. Nevertheless, a Lyosphere was introduced onto the cartridge in the elution chamber, and water was used for rehydration. The Lyosphere was fully and uniformly rehydrated in less than 10 seconds.

Sample-to-answer testing on the integrated system: Sample-to-answer testing was performed for all relevant microfluidic steps associated with an end-to-end result. The sequence of steps that were successfully integrated and automated using the microfluidic cartridge after addition of a blood sample were: introduction of bind buffer into the sample tower, mixing sample with bind buffer using air, transport of sample mix to TruTip tower, toggle between the towers, dispense to waste, introduction of wash buffer to the TruTip tower, toggle between the towers, dispense to waste, introduction of elution buffer, and dispense to elution tower, add PCR mix to elution tower, dispense to PCR chamber on flow cell, and thermal cycle with bladder thermal cycler, flush PCR chamber with APEX buffer and add to APEX reservoir, thoroughly mix, add APEX reagent to array chamber, incubate at 65°C, wash, and image. We were able to demonstrate this using one SNP marker and not all six SNPs since the assay

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development required more time than planned and further assay and hardware improvements are required to improve signal intensities on the array. Imaging was performed off the system because further sensitivity enhancements were deemed to be necessary. These improvements may be achieved by using lower autofluorescence materials for the microarray chamber, increased sensitivity for the CCD camera, and/or more efficient amplification and/or APEX reactions.

Implications for Policy and Practice

Phase III will continue defining protocols and assay chemistries, add additional SNP markers, and further refine the packaging of components and reagents in the integrated system. Plans are in progress to reconfigure the platform in Phase III to allow processing and analyzing up to 8 samples in parallel. Low cost molding of the disposables will be more cost-effective in the updated design. Developing an intuitive software and graphical user interface, and reducing the instrument footprint (approximately 2-3 cubic ft) remain high priorities. Protocols and assays will further be streamlined for minimum complexity, time, and cost. In addition, we look to complete transition assay reagents (*e.g.*, PCR, APEX) into the lyophilized format for long term storage and field deployment.

The successful commercialization of an end-to-end SNP-typing platform has important implications for translating forensically important physical appearance DNA markers into a low-cost, user-friendly format. The most noteworthy is the unreliability of eyewitnesses. Our platform could confirm or reject an eyewitness description. The major benefit would be preventing misdirection of law enforcement resources by inaccurate eyewitness descriptions of a missing suspect or victim.

Given the fluidic process described herein, the same basic microfluidic format and manufacturing infrastructure could be applied to 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; 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. 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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MAIN REPORT

I. INTRODUCTION

Statement of the Problem

One of the main requirements to apprehend a criminal or identify a victim is the determination of the individual's physical appearance. Identification is often possible using witnesses, friends and family, colleagues, etc. However, many crimes are often associated with missing bodies, highly degraded bodies, trace samples of blood or semen, body parts, partial skeletal remains or pieces, or have no or unreliable witnesses. In these cases, forensic scientists utilize more sophisticated approaches to aid in identification, including fingerprints, dental records, hair and skin analyses, skeletal reconstruction/modeling, and DNA typing. DNA typing, while very powerful, is only useful in these kinds of cases if a match is obtained in CODIS or with a possible reference sample (*e.g.*, toothbrush, family member). When the physical evidence is minimal, information on an individual's identity and physical appearance is often limited, thus making the case more difficult to solve. Therefore, any new tool to make this process easier, faster, and more accurate, would be valuable to the criminal justice community.

It is well-known that a person's physical appearance is determined predominantly by their genome. With the advent of bioinformatics deciphering the wealth of biallelic SNP genotyping data, a powerful new application for SNPs in forensics is emerging. This new application, in which STRs are not useful, is the prediction or determination of an unknown individual's physical appearance with only a trace DNA sample [1]. The physical traits revealed by SNPs can be as simple as eye, hair, and skin color, but can get more sophisticated and include height, facial features, hair type and growth pattern, etc. Currently, the widely used STR kits include the amelogenin deletion (non-STR marker) to determine an individual's sex phenotype. Our approach will greatly expand this information.

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 provide a new tool to aid in apprehending a perpetrator or identifying a victim, and reducing the number of cold cases. It is our assertion that the Akonni technology offers such advantages.

Literature citations and review

SNPs for physical appearance: Over the last few years, there has been a tremendous understanding of the linkage of human physical appearance to SNP genotypes. While there can be many SNPs associated with a specific aspect of physical appearance, such as eye color, the SNPs can be down selected to only a few key genetic predictors. Table 1 list six SNPs selected by Liu *et al*. (2008) [2] that were determined as major genetic markers for eye color, with an overall AUC (integral of the receiver operating characteristic curves) of 0.93 for brown, 0.91 for blue, and 0.72 for intermediate colored eyes. Hair color can be also associated with some of these SNPs [3] as noted in the table. Since there is substantial sequence information available for the six eye color SNPs, they served as the basis for our model assay in Phase II.

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Table 1. Six SNPs Identified as Major Determinants for Eye Color [2]

SNP-ID	Chr	Position	Gene	Common Allele/ Eye color	Minor Allele/ Eye color	Notes
rs12913832	15	26039213	HERC2	G / Blue	A / Brown	
rs1800407	15	25903913	OCA2	C/ Brown	T/ Blue	T/ associated with Green/hazel
rs12896399	14	91843416	SLC24A4	T/ Blue	G/ Brown	
rs16891982	5	33987450	SLC45A2	G/ Blue	C/ Brown	C/ associated with Black hair
rs1393350	11	88650694	TYR	G/ Brown	A/ Blue	
rs12203592	6	341321	IRF4	C/ Brown	T/ Blue	

Current technologies

As listed in Table 2 [4], there are many companies commercializing technologies for SNP analysis. However, no company offers a complete solution that: 1) automates the total analysis process, including sample preparation, 2) meets LOD requirements for low copy template (many of the technologies are well-suited for high copy template for standard genetic testing), 3) provides a small footprint instrument, 4) exhibits relatively short total analysis times, and 5) meets the cost requirements for a low budget facility. Therefore, there remains a need to develop an instrument that can offer a complete solution for the forensic laboratory.

Table 2. Companies offering genotyping products [4]

Company	Web address	Company	Web address
454 Life Sciences	http://www.454.com	Illumina	http://www.illumina.com
ACGT Inc.	http://www.acgtinc.com	GenScript Corp.	http://www.genscript.com
Affymetrix	http://www.affymetrix.com	IMGM Laboratories	http://www.dap-healthcare.com
Agencourt Bioscience	http://www.agencourt.com	Invitrogen	http://www.invitrogen.com
Agilent Technologies	http://www.agilent.com	KBioscience	http://www.kbioscience.co.uk
Applied Biosystems	http://www.appliedbiosystems.com	Lab. Corp. of America	http://www.labcorp.com
ATLAS BioLabs	http://www.atlas-biolabs.de	Marligen Biosciences	http://www.marligen.com
Beckman Coulter	http://www.beckmancoulter.com	MiraiBio	http://www.miraibio.com
BioDiscovery	http://www.biodiscovery.com	Nanogen	http://www.nanogen.com
BioTrove	http://www.biotrove.com	Ocimum Biosolutions	http://www.ocimumbio.com
Bruker Daltonics	http://www.bdal.com	Orchid Cellmark	http://www.orchidbio.com
Cogenics	http://www.cogenics.com	PerkinElmer	http://las.perkinelmer.com
deCODE Genetics	http://www.decode.com	Perlegen Sciences	http://www.perlegen.com
Ellipsis Biotherapeutics	http://www.ellipsisbio.com	Plexigen	http://www.plexigen.com
Enzo Life Sciences	http://www.enzo.com	Precision Biomarker Resources	http://www.precisionbiomarker.com

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EpigenDx	http://www.epigendx.com	Premier Biosoft	http://www.premierbiosoft.com
Expression Analysis	http://www.expressionanalysis.com	Progeny Software	http://www.progenygenetics.com
Fluidigm	http://www.fluidigm.com	Promega	http://www.promega.com
Geneservice	http://www.geneservice.co.uk	Qiagen	http://www.qiagen.com
GeneWorks	http://www.geneworks.com.au	Roche Applied Science	http://www.roche-applied-science.com
Genizon Biosciences	http://www.genizon.com	Roche Nimblegen	http://www.nimblegen.com
GenoLogics	http://www.genologics.com	Sequenom	http://www.sequenom.com
GenScript Corp.	http://www.genscript.com		

Most of the technologies offered by the companies in Table 2 can be categorized in Table 3 [5]. Table 3 lists the allelic discrimination reaction (assay) and how it can be implemented with a mode of detection (hardware) of the assay product. For example, the commercially available ABI SNPlex and Illumina Veracode instrumentation for SNP discrimination are both based on oligonucleotide ligation assays. The ABI SNPlex utilizes capillary electrophoresis detection and the Illumina Veracode makes use of a bead-based array detection. These tests involve probes that directly interrogate the genomic DNA, and do not use PCR. These platforms can work well for high copy template samples (such as reference samples), but are inadequate for the often encountered low copy template or degraded forensic samples. Our proposed effort includes a PCR step for the integrated system.

Table 3. Compatible Allelic Discrimination Reactions and Detection Methods [5]

Detection Method / Allelic Discrimination Reaction	Electrophoresis (fluorescence)	FRET	Fluorescence polarization	Arrays (fluorescence)	Mass spectrometry	Luminescence
Allelic-specific hybridization		✓	✓	✓		
Primer extension	✓	✓	✓	✓	✓	✓
Oligonucleotide Ligation	✓	✓		✓		
Invasive cleavage		✓	✓		✓	

The ABI SNaPshot, a highly studied SNP discrimination kit for forensics, is a primer extension assay analyzed using a capillary electrophoresis (CE) instrument, such as the ABI 310 Genetic Analyzer. The ABI 310 Genetic Analyzer does not do the sample preparation and PCR steps, yet it costs over $20,000 and weighs over 200 lbs. Ten-plex and higher multiplexes have been demonstrated on the SNaPshot and CE platform. However, it is a significant effort with the SNaPshot to balance the concentrations and interactions of the extension primers in solution to produce a robust assay. The AS-APEX assay, as proposed for our effort (denoted by green circle in the Table 3), makes high multiplex primer extension assays less challenging, since the primers are spatially separated during the extension reaction, thereby eliminating primer interaction interferences. In addition, AS-APEX is an established and effective approach for low to medium density arrays [9-12].

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Most importantly, we have developed a new front-end sample preparation technology, the TruTip, that is very compatible with our integrated cartridge concept. Sample preparation is often trivialized in new instrumentation development, especially with respect to approaches that are integration friendly and compatible with real-world volumes (e.g., 0.5-1.0 ml swab extracts). With our expertise in fluidics and system integration, we offer a complete solution for a total analysis platform for commercialization.

Phase I Akonni Technologies

Gel Element Microarray: Akonni Biosystems has an exclusive license to a mature gel element array intellectual property portfolio originally developed at Argonne National Laboratory in collabortion 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.*, [10-20]). The fundamental difference between Akonni's 3-dimensional gel element arrays and other commercially available substrates is that individual polymeric gel elements create a high density array of 3-dimensional "test tubes". Probes are covalently crosslinked to the polymer backbone instead of the solid substrate. Thus, each gel element retains a solution-phase test environment throughout manufacturing and testing, and biomolecular interactions proceed according to well understood, liquid-phase thermodynamics and kinetics without uncharacterized or unknown surface effects that plague conventional microarray substrates. The gel element array is surrounded by proven and deployed sample preparation chemistries, portable microarray imaging equipment, and automated analysis and decision software 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).

AS-APEX for SNP Typing: AS-APEX method has been previously described in the literature by others to discriminate single nucleotide differences from amplified PCR products on a microarray [6-9]. 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 result is a more streamlined APEX method that is faster and just as effective.

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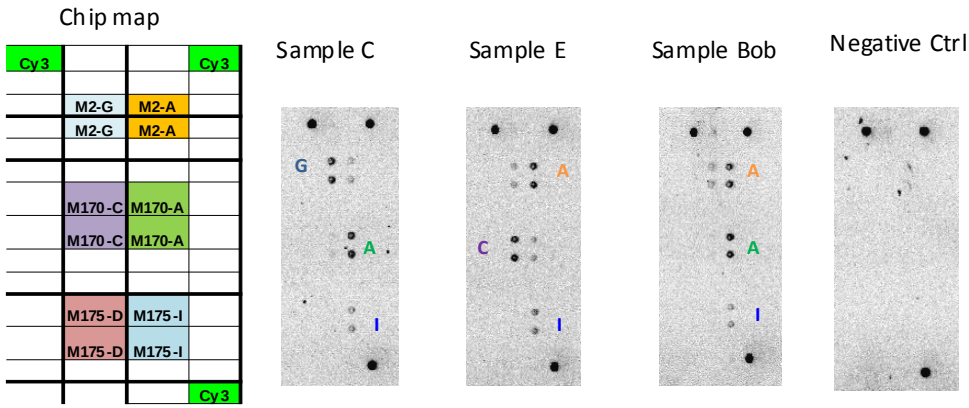

Figure 1. Visual analysis of multiplexed AS-APEX assays on Sample C, E, "Bob" and negative control. The chip map on the left indicates the assay (M2, M170, or M175) and the 3′ base of the allele-specific primer (G, A, or C). D and I denote deletion (or lack of insertion) and insertion, respectively. Sample "Bob" is a semen sample with the genotype A, A, I for M2, M170, and M175, respectively.

We have designed our primers so that a single primer is immobilized within 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. In the presence of the correct target and matched 3' base, polymerase incorporates fluorescently-labeled nucleotides to produce the final signal. In Phase I, we applied this strategy to the detection of three Y-chromosome SNPs (M2, defining most African Americans, M170, defining most Caucasians, and M175, defining most Asians). Figure 1 shows the array images for several Y-chromosome typed DNA samples. The correct genotype is visually apparent for each sample.

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 2 (left panel) shows the correct typing results for both APEX reactions, demonstrating sensitivity down to 100pg or roughly 16 genomic copies.

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

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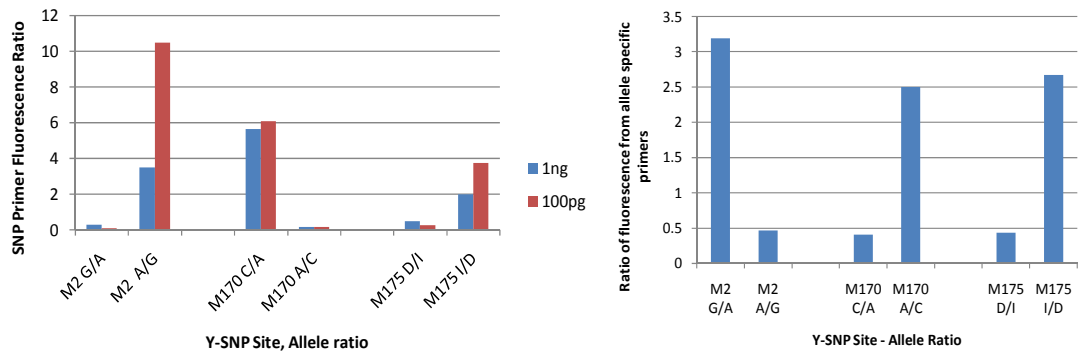

Figure 2: AS-APEX for Y-chromosome markers has a sensitivity of 100 pg or 16 genomic copies, even in a high background of 10 ng of female DNA. (left) Allele specific primer ratios for multiplex PCR amplified NIST samples starting with 1ng (blue) or 100pg (red) genomic DNA. (right) Allele specific primer ratios for APEX assay using PCR product from 10ng female and 100pg male DNA. Results are an average of four replicates. The SNP primer fluorescent ratio is the fluorescent signal of the primer extended by one of the alleles divided by the signal of the primer extended by the other allele. Both ratios are presented, with the higher ratio value indicating the haplotype allele that is present. For example, the genotype determined for the left panel is A, C, I (insertion) for loci M2, M170, and M175, respectively.

Sample Preparation Subcircuit –TruTip: For sample preparation, Akonni uses a modified pipet tip (called TruTip) containing a rigid, porous silica extraction matrix to bind and elute nucleic acid (Figure 3, upper left). The matrix allows bi-directional flow for improved binding and elution efficiencies. It accommodates a wide range of sample volumes (*e.g.*, 1-1000ul), with the larger volumes necessary for extractions from swabs. In the case of crime scene samples, the swabs may contain low copy template and be subjected to concentration using a low porosity spin filter (*e.g.*, Centricon) and/or centrifugation. The TruTip eliminates the need for these steps. Most noteworthy is the ubiquitous nature of the TruTip form factor, which allows implementation using a single or a multi-channel pipet, a robotic pipet workstation, or as a subcircuit in our microfluidic cartridge.

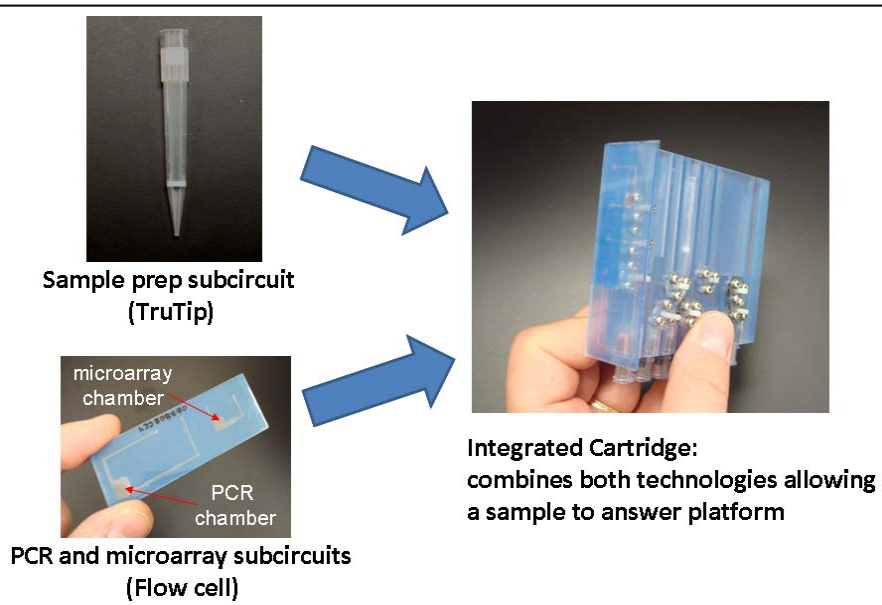

Figure 3. Working subcircuits developed during Phase I were integrated into a cartridge for Phase II.

Comparisons of the quantitative real-time PCR results obtained using the Akonni's TruTip (operated by Rainin Electronic Pipettor) and a standard Qiagen kit indicated

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that both methods exhibited comparable efficiency and recovery (Figure 4). In addition, TruTip was faster, utilizing: 1) a 30 min lysis 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. Other results provided to us by BioHelix, an independent evaluator of the TruTip product, showed that the TruTip demonstrated a 60-70% recovery of Neisseria gonorrhoeae from 500 ul of urine using only a four minute protocol (data not shown). In Phase II, we are proposing to test a bead-blender as a replacement for the lysis buffer. In addition, we have developed our own formulation of the lysis buffer that only requires a 5-minute incubation for 100-ul whole blood samples (data not shown).

The TruTip and Qiagen kits were also evaluated on saliva samples. Real-time PCR quantitation of processed triplicate samples yielded 46.27ng/ul for Qiagen and 59.1ng/ul for the TruTip (using the blood 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 and 6ug for the TruTip.

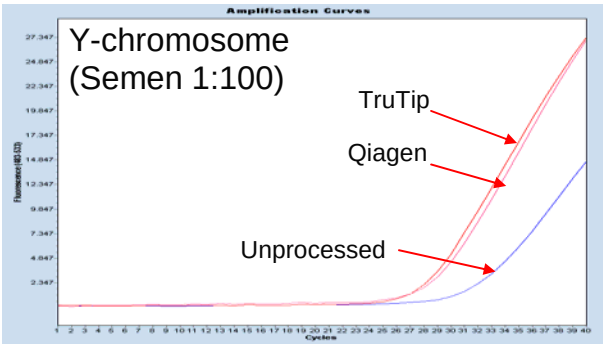

Figure 4. The TruTip device provides the same sample preparation performance as the commercial Qiagen kit. Both methods included a lysis step using the Qiagen AL lysis buffer prior to extraction. The incubation time was 30 min for the TruTip and 60 min for the Qiagen kit

To automate the TruTip process, a sample preparation subcircuit (Figure 5) was designed that consisted of a machined prototype cartridge with inlets, outlets, and a receptacle for attaching a TruTip. The TruTip is disposable and replaced after each run. Although the machined cartridge and other liquid handling components that the sample comes in contact are presently reusable for rapid prototyping and protocol development, 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. The pipet tip form factor will be maintained in the integrated cartridge that contains the sample preparation, PCR, and microarray subcircuits.

In Phase I, the cartridge was connected to the Flow Control Station, developed by Global FIA, as shown in Figure 5 (right panel). The Flow Contols Station contained a milligat pump, multi-port valves, and the bulk reagents. The Flow Control Station metered and moved fluids from the bulk reagents to the cartridge. Protocols optimized using the Rainin pipettor were converted to scripts on the Flow Contol Station. 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. Additionally, one-way valves made cartridge insertion and removal simple and easy without the risk of losing sample due to leakage after the process was completed.

Three semen samples were sequentially processed on the automated system. In the protocol, the semen sample was added to the holding tube (which will be performed in the cartridge in Phase II) that also contained 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 as harsh decontamination procedures were not implemented since there was uncertainty on how harsh decontamination solutions would react to some of the

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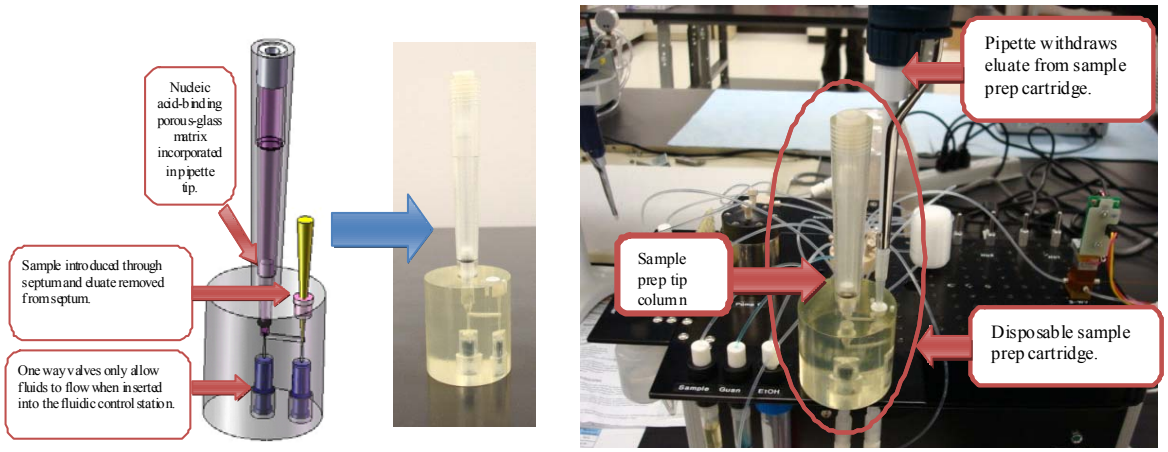

Figure 5: (left) Design and photograph of the prototype of sample prep cartridge. The sample was introduced through a septum via a pipettor that seals after removal of the tip. (right) Prototype sample preparation subcircuit, containing the cartridge connected to the Flow Control Station to demonstrate automated nucleic acid purification from semen. Also shown is the pipettor withdrawing the eluate following the automated purification.

materials, and as the ultimate goal is to confine the sample to a one-time-use disposable. As shown in Figure 6 (right panel), the three replicates had better CTs than that of the unprocessed sample, indicating that the purification and concentration was successful. Some carryover contamination was evident as indicated by a signal from the water flush, but this was not a significant contributor to the signals obtained from the eluted fractions. When a 10-fold higher sample of semen was tested (Figure 6, left panel), the PCR signal from unprocessed semen was highly inhibited, whereas the processed semen sample was uninhibited, displaying a strong positive PCR signal.

Carryover was eliminated in Phase II as low-cost disposable valves were incorporated into the cartridge to retain all sample and sample movement within the cartridge after the cartridge is engaged with the docking station. In addition, the cartridge is intended to be a one-time use disposable.

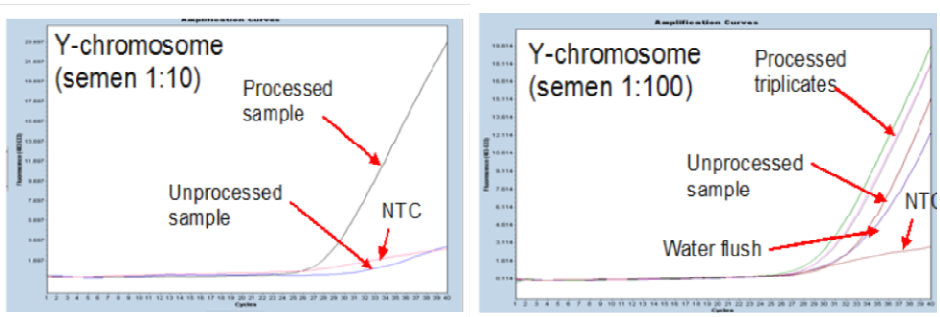

Figure 6. Successful processing of semen on the sample preparation circuit prototype.

PCR and Micrarray Subcircuits - Flow Cell: For PCR and microarray reaction chambers, Akonni employs a flow cell (Figure 3, lower left) consisting of proprietary materials and favorable fluidic properties. The flow cell allows filling and emptying the chambers without bubble formation. The materials are PCR-friendly, exhibiting no leaching of inhibitors into the reaction or adsorption of the polymerase to the chamber walls. Various iterations of subcircuits were designed and tested. The subcircuit utilized two chambers, one for PCR and the other for APEX. The APEX chamber contained a gel-spot microarray.

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For Phase II, these Akonni advancements were integrated into a unique microfluidic cartridge (Figure 3, right) that maintains the TruTip and TruArray flow cell form factor. The cartridge minimized fluidic circuitry and complexity while introducing embedded pin valves.

Bladder Thermal Cycler.

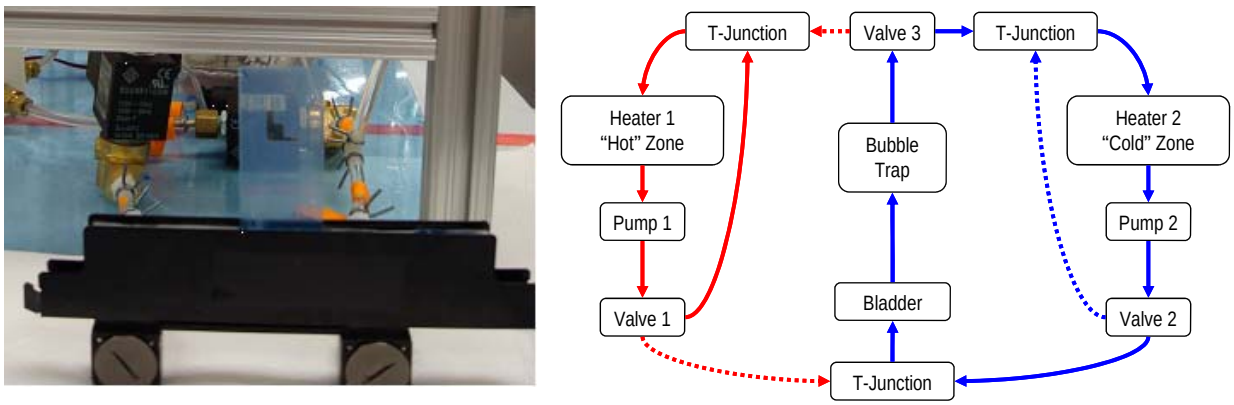

Figure 7. (left) Flow cell (separate PCR and microarray chambers) with PCR chamber inserted into the bladder themal cycler. (right) Flow diagram of the dual-loop circulation system in the bladder thermal cycler. Depicted is fluid from the cold zone (blue) being directed through the bladder for the annealing 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.

A bladder-based thermal cycler making use of a temperature-controlled bladder pair was developed as a preferred means to interface thermal cycler heaters with the PCR chamber in the flow cell (Figure 3, lower left). When the bladders are relaxed, it is easy to position the flow cell, or cartridge, between the bladder pair. When the bladders are pressurized, the bladders tightly press against the walls of the flat reaction chamber of the subcircuit, and the bladder thermal cycler alternately gates two temperature-controlled fluids through the bladders to generate thermal cycling conditions.

Figure 7 (left panel) shows a flow cell with the PCR chamber inserted between a bladder pair. The flow cell was subjected to thermal cycling based on a dual-loop circulation system as illustrated in Figure 7 (right panel). This circulation system maintained two temperature zones of circulating fluids. Each zone had a dedicated heat exchanger and pump. Three 3-way valves were used to divert the desired temperature fluid through the bladder pair. Re-circulating fluids were 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.

Figure 8 shows temperature profiles of a thermal cycling run performed with the dual-loop bladder thermal cycler with set points of 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

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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. We made improvements to the thermal cycler by PID controlling the flow rate through the heaters and bladders during Phase II.

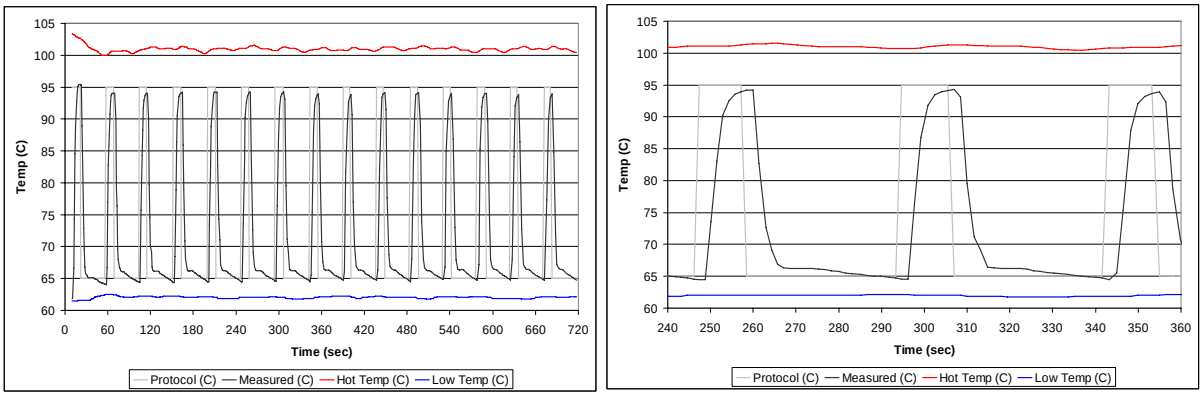

Figure 8. Temperature profiles generated from the bladder thermal cycler. The heating and cooling rates averaged 13.6°C/s and 17°C/s, respectively. 40 cycles of thermal cycling on the flow cell was completed in about 40 minutes.

Conventional thermal cyclers that perform heating and cooling at a fixed site achieve fast 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 [21-24]. Our dual-loop circulation in conjunction with the bladder heaters permits simplified temperature control while keeping the reaction stationary at a single site.

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 and replace the mechanics (*e.g.*, clamps, pneumatics, etc.) required to interface rigid heaters to a rigid flat, wall of a PCR chamber in an integrated, disposable cartridge.

Sample-to-Answer Demonstration in Phase I: As a first step in a sample to answer test, a semen sample was processed by TruTip extraction on the testbed cartridge, the eluted sample was amplified by

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multiplex PCR in a tube on the MJ thermal cycler, the product was subjected to AS-APEX for SNP discrimination, and the fluorescent signals on the microarray were imaged on the reader. The results shown in Figure 9A demonstrate correct allele detection and identification among the three Y-chromosome markers.

The highlight of Phase I was using all the components planned for Phase II, in which the same semen sample was processed by automated TruTip extraction on the testbed cartridge; the eluted sample was amplified in the PCR chamber in the flow cell positioned in the bladder thermal cycler; the product was subjected to AS-APEX for SNP discrimination; and the fluorescence signal on the microarray was imaged. The results in Figure 9B show that the correct genotyping was obtained for the sample to answer demonstration.

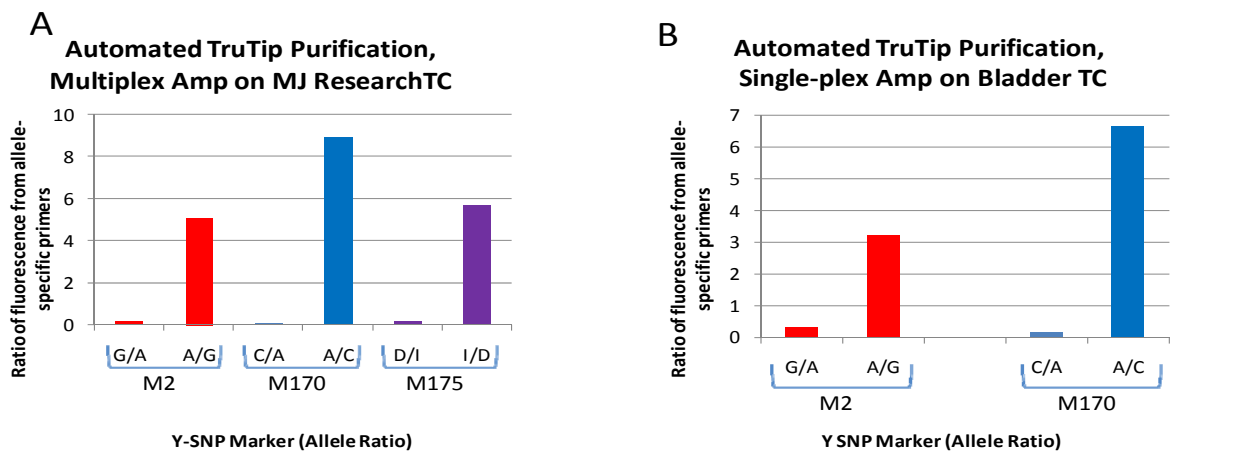

Figure 9. Allele specific primer ratios for (A) multiplexed or (B) single-plex AP-APEX assays for a processed sample of semen resulting in correct genotyping. The SNP primer fluorescent ratio is the fluorescent signal of the primer extended by one of the alleles divided by the signal of the primer extended by the other allele. Both ratios are presented, with the higher ratio value indicating the haplotype allele that is present. For example, the genotype determined for the Panel A is A, A, I (insertion) for loci M2, M170, and M175, respectively.

Statement of hypothesis or rationale for the research

Elucidating an individual's appearance (*e.g.*, eye color, hair color, height, etc.) from a forensic sample can provide critical information to identify a suspect (especially if an STR profile is not in the CODIS database) or a victim (*e.g.*, samples could include blood, highly degraded remains, body parts, or other trace evidence). Thus the rationale for Phase II was to integrate and package components successfully demonstrated in Phase I to type six SNPS that are closely associated with eye color as the model assay.

II. METHODS

ASSAY DEVELOPMENT

Design of SNP-specific primers for array extension: SNP-specific primers targeting different SNPs (Table 1) were designed using Oligo 6 software. Generally these primers were designed to be 20-23

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bases in length, and the 3′ base of the primer is the SNP site. Different primer lengths were tested. A list of these primers is presented in Table 4.

Table 4. List of SNP-specific primers used for APEX

SNP-ID	Sequence 5'-3'	SNP Primer ID
rs12913832	5'-MET-C18-TAGCGTGCAGAACTTGACAT	A-rs12913832_Dn_T20
	5'-MET-C18-TAGCGTGCAGAACTTGACAC	A-rs12913832_Dn_C20
	5'-MET-C18-ATAGCGTGCAGAACTTGACAT	A-Rs12913832_Dn_T21_NW
	5'-MET-C18-ATAGCGTGCAGAACTTGACAC	A-Rs12913832_Dn_C21_NW
rs1800407	5'-MET-C18-GCATACCGGCTCTCCCG	A-Rs1800407_Up_G17
	5'-MET-C18-GGCATACCGGCTCTCCCA	A-Rs1800407_Up_A17
	5'-MET-C18-CATACCGGCTCTCCCG	A-Rs1800407_Up _G16_NW2
	5'-MET-C18-CATACCGGCTCTCCCA	A-Rs1800407_Up _A16_NW2
	5'-MET-C18-ATACCGGCTCTCCCG	A-Rs1800407_Up _G15_NW3
	5'-MET-C18-ATACCGGCTCTCCCA	A-Rs1800407_Up _A15_NW3
rs12896399	5'-MET-C18-TTT AGG TCA GTA TAT TTT GGG G	A-rs12896399_Up_G22
	5'-MET-C18-CTT TAG GTC AGT ATA TTT TGG GT	A-rs12896399_Up_T23
	5'-MET-C18-TCTTTAGGTCAGTATATTTTGGGG	A-Rs12896399_Up_G24_NW
	5'-MET-C18-TTCTTTAGGTCAGTATATTTTGGGG	A-Rs12896399_Up_G25_NW
	5'-MET-C18-GTTCTTTAGGTCAGTATATTTTGGGG	A-Rs12896399_Up_G26_NW
	5'-MET-C18-TCTTTAGGTCAGTATATTTTGGGT	A-rs12896399_Up_T24_NW
	5'-MET-C18-TTCTTTAGGTCAGTATATTTTGGGT	A-rs12896399_Up_T25_NW
	5'-MET-C18-GTTCTTTAGGTCAGTATATTTTGGGT	A-rs12896399_Up_T26_NW
rs16891982	5'-MET-C18-AAAACACGG AGTTGATGCAC	A-Rs16891982_Up_C20
	5'-MET-C18-AAAACACGG AGTTGATGCAG	A-Rs16891982_Up_G20
rs1393350	5'-MET-C18-CAGTCCCT TCTCTGCAACG	A-Rs1393350_Up_G19
	5'-MET-C18-TCAGTCCCT TCTCTGCAACA	A-Rs1393350_Up_A20
	5'-MET-C18-TCAGTCCCTTCTCTGCAACG	A-Rs1393350_Up_G20_NW
	5'-MET-C18-CTCAGTCCCTTCTCTGCAACG	A-Rs1393350_Up_G21_NW
	5'-MET-C18-CCTCAGTCCCTTCTCTGCAACG	A-Rs1393350_Up_G22_NW
	5'-MET-C18-CTCAGTCCCTTCTCTGCAACA	A-Rs1393350_Up_A21_NW
	5'-MET-C18-CCTCAGTCCCTTCTCTGCAACA	A-Rs1393350_Up_A22_NW
	5'-MET-C18-GAAAACACGGAGTTGATGCAC	A-Rs16891982_Up_C21_NW
	5'-MET-C18-GAAAACACGGAGTTGATGCAG	A-Rs16891982_Up_G21_NW
rs12203592	5'-MET-C18-CTTTGGTGGGTAAAAGAAGGC	A_rs12203592_Up_C21
	5'-MET-C18-CTTTGGTGGGTAAAAGAAGGT	A_rs12203592_Up_T21

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	5'-MET-C18-ACTTTGGTGGGTAAAAGAAGGC	A_Rs12203592_Up_C22_NW
	5'-MET-C18-CACTTTGGTGGGTAAAAGAAGGC	A_Rs12203592_Up_C23_NW
	5'-MET-C18-ACTTTGGTGGGTAAAAGAAGGT	A_Rs12203592_Up_T22_NW
	5'-MET-C18-CACTTTGGTGGGTAAAAGAAGGT	A_Rs12203592_Up_T23_NW

Design of multiplex PCR primers: Sequences for primers for multiplex PCR were taken from [2] except for primers for rs12203592 which were designed in-house. Selected primers generated short amplicons to minimize the influence of secondary structures on hybridization efficiency and avoid the necessity for a fragmentation step prior to APEX. In addition, a second set of primers were designed to produce longer amplicons that could be used for sequencing the SNP regions. A list of the primers is presented in Table 5 (note that for rs12203592 one and the same primer pair was used for to produce amplicons for APEX and for sequencing).

Table 5. Primers for multiplex PCR and sequencing.

ID	Sequence; 5'-3'	Position/Length	Amplicon Length
Primers for Multiplex PCR for APEX [2]			
rs1393350 F	TACTCTTCCTCAGTCCCTTC	rs1393350:273U20	84
rs1393350 R	GGAAGGTGAATGATAACACG	rs1393350:337L20	
rs16891982 F	AAAGTGAGGAAAACACGGAG	rs16891982:273U20	66
rs16891982 R	TCTACGAAAGAGGAGTCGAG	rs16891982:319L20	
rs1800407 F	ACTCTGGCTTGTACTCTCTC	rs1800407:248U20	81
rs1800407 R	ATGATGATCATGGCCCACAC	rs1800407:309L20	
rs12913832 F	CGAGGCCAGTTTCATTTGAG	rs12913832:225U20	79
rs12913832 R	AAAACAAAGAGAAGCCTCGG	rs12913832:284L20	
rs12896399 F	TCTGGCGATCCAATTCTTTG	rs12896399:457U20	78
rs12896399 R	GATGAGGAAGGTTAATCTGC	rs12896399:515L20	
Primers for sequencing PCR			
rs1393350 F_SEQ	CCTGGACATCATTTTCTCACTG	rs1393350:211U22	147
rs1393350 R_SEQ	GGGAAGGTGAATGATAACACG	rs1393350:337L21	
rs16891982 F_SEQ	ACCAGAAACTTTTAGAAGACATCC	rs16891982:222U24	144
rs16891982 R_SEQ	GTGCACACAACTCCACAGAG	rs16891982:346L20	
rs1800407 F_SEQ	AAAGGCTGCCTCTGTTCTAC	rs1800407:218U20	148
rs1800407 R_SEQ	CAAGAAGGCAGAGAGGACG	rs1800407:347L19	
rs12913832 F_SEQ	ACAAAGGTACAGGAACAAAGAA	rs12913832:166U22	138
rs12913832 R_SEQ	AAAACAAAGAGAAGCCTCGG	rs12913832:284L20	
rs12896399 F_SEQ	CCTATATTTTATCTGGCGATCC	rs12896399:446U22	116
rs12896399 R_SEQ	CTTAGCCCTGGGTCTTGAT	rs12896399:543L19	

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rs12203592 F_SEQ	CAGCTGATCTCTTCAGGCTTTC	rs12203592:179U22	137
rs12203592 R_SEQ	ACTGTTTTATGTAAAGCTTCGTCA	rs12203592:292L24	

Synthesis of primers and oligonucleotide probes for TruArray: Oligonucleotide synthesis was peroformed on an ABI 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.

Genomic DNA samples: Genomic DNA used was: NIST SRM 2395, NIST SRM 2372, de-identified samples from our collaborator the Massachusetts State Police. Blood was purchased from Valley Biomedical and semen was purchased from Xytex Corp.

Fabrication of TruArrays: Oligonucleotides were dissolved at 100 µM concentration in Akonni's polymerization mixture and transferred to the wells of a 384-well microtiter 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.

PCR: Typically PCR was carried out in 50 µl volumes containing 0.5-10 ng of genomic DNA, 400 nM of reverse primers (except for rs12913832) (Cy3-labeled primers were used for producing amplicons for hybridization test) and 40 nM of primers (except for rs12913832) in 1×Qiagen Multiplex PCR mix (Qiagen, Valencia, CA, US) with 0.5xQ-solution (Qiagen), 2.5-3.75% formamide, 2-4 units of Taq Polymerase (Qiagen) and 0.25 ug ET SSB (Extreme Thermostable Single-Stranded DNA Binding Protein; Biohelix). Amplification was carried out in MJ PTC-225 thermal cycler. Thermal cycling parameters for low-temp PCR included initial denaturing at 85°C for 3 min; 50 cycles of 30 s at 85°C, 60s s at 56°C, and a final extension at 56°C for 3 min.

Hybridization: Hybridization with produced amplicons was carried out in order to test the presence of the amplicons. Biochips for hybridization contained limited set of SNP-specific primers from Table 1. 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 Frame-Seal chambers (Biorad) in MJ PTC-225 with Twin Tower block for slides. Hybridization was carried out at 50°C for 3h. Following the hybridization,

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the chambers were removed and the slides with the arrays 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.

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.

AS-APEX procedure: Briefly, for a 60µL reaction, 6 µL of amplified target (from a multiplex PCR amplification with 1-10ng DNA target) 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/dTTP (NEB), 1µM Alexa-546-dUTP (Invitrogen), 1 µM Cy-dCTP (GE HealthCare) and 5U Deep Vent Exo- polymerase (NEB). The sample was applied to the APEX chamber formed by a double layers of 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-225 thermal cycler set to the 65°C. After incubation, 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 5 seconds.

Lyophilized reagents: Lyophilized pellets (Lyospheres) for PCR and APEX were produced at Biolyph (http://www.biolyph.com/) according to formulations developed at Akonni. At the first round of lyophlization experiement, complete PCR and APEX mixes, which included all reaction components, were lyophilized with three different excipient recipes. At the second round of lyophilization experiments, for evaluation of the effect of lyophilization process on different reaction parameters, PCR buffer, Taq Polymerase, APEX reaction buffer, and Deep Vent Exo- Polymerase were lyophilized independently. In addition, each reaction component was lyophilized with three different excipient recipes (2S, 4S, 7S) in order to study lyophilization and reaction performances.

PCR buffers for lyophilization included our routine buffer on the basis of Qiagen Multiplex Mix as well as alternative PCR buffer on the basis of standard Qiagen PCR buffer which is supplied with Qiagen Taq Polymerase. PCR buffers for lyophilization did not contain formamide. Enzyme components for lyophilization were subjected to dialysis using Slide-A-Lyzer Mini Dialysis units (Thermo Scientific) in order to remove glycerol from storage buffer to ensure good lyophilization efficiency.

Lyophilization of Qiagen Multiplex Mix produced relatively good pellets with all excipient recipes (2S, 4S, and 7S). However, lyophilization of the PCR buffer of the basis of standard Qiagen PCR buffer produced somewhat poor results, pellets with questionable quality were obtained only with 4S excipients. After discussion with Biolyph, we hypothesize that this could be caused by the presence of Q-solution (which contains betaine) in the reaction mix. However, the question remains why the same concentration of Q-solution did not cause such problems for Qiagen Multiplex Mix buffer. Lyophilization of Qiagen Taq Polymerase also produced good results for all excipient recipes.

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Lyophilization of Deep Vent Exo- produced acceptable pellets only for 2S excipients. We hypothesize that this could be caused by residual glycerol remaining in the Deep Vent solution after dialysis, *i.e.* for some reason the efficiency of dialysis was worse for Deep Vent Exo- in comparison with Taq Polymerase.

Lyophilized pellets (LyoSpheres) were used to produce PCR mixes for singleplex and multiplex amplifications and APEX reaction mixes. Each lyophilized component was tested separately (*e.g.*, lyophilized PCR buffer was combined with fresh Taq Polymerase or vice versa) and in combination with another lyophilized component (*e.g.*, lyophilized PCR buffer with lyophilized Taq Polymerase). Fresh non-lyophilized mixes were used as controls.

HARDWARE DEVELOPMENT

The integrated system consisted of an integrated disposable cartridge and an instrument.

Integrated Cartridge

The integrated cartridge (Figure 10) consisted of an embedded TruTip for nucleic acid purification, cartridge pin valves to direct reagents to specific locations on the cartridge, a thin film flow cell for rapid PCR and for APEX detection, and a sealed lid to provide a fully-contained system. The cartridge pin valves were inserted into a cavity on the cartridge and sealed in place with holders. The actuators in the instrument closed and opened the pin valves.

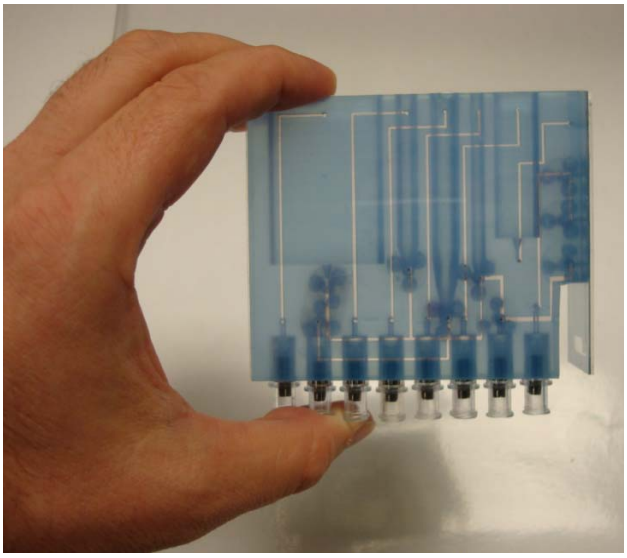

Figure 10. Integrated cartridge with attached flow cell on right.

The sequence of steps for sample prep on the cartridge were: introduction of sample into sample tower, introduction of bind buffer into sample tower, mixing with air, transport of sample mix to TruTip tower, toggle between the towers, dispense to waste, introduction of wash buffer to the TruTip tower, toggle between the towers, dispense to waste, introduction of elution buffer, and dispense to elution tower. The sequence of steps for PCR were: add PCR mix to elution tower or reconstitute Lyosphere (lyophilized

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reagent pellet) with eluent, dispense to PCR chamber on flow cell, and thermal cycle. The sequence of steps for APEX were: flush PCR chamber with APEX buffer and add to APEX reservoir, thoroughly mix, add APEX reagent to array chamber, incubate at 65°C, wash, and image.

The Instrument

The instrument consisted of three subsystems: Fluidic, Thermal Cycler and Optical.

Fluidic Sub-System: The Fluidic Sub-System consists of three types of functional components: bidirectional microfluidic pumps, selection valves, and cartridge "pin" valves. The bidirectional microfluidic pumps and selection valves are sourced from Global FIA. Also, part of the system is a manifold, shown in Figure 11, that connects fluidic lines from the cartridge to the pumps and valves. The manifold allows two ¼-28 fluidic fittings to be connected for each cartridge port. The manifold has eight bosses with luer tapers. These bosses have a fluidic channel that provides a flow path from the fluidic fittings to the cartridge port.

Figure 11. Machined manifold that accepts cartridges and introduces buffers/reagents to the cartridge.

The Fluidic Sub-System includes two bidirectional pumps: one for sample prep and one for PCR/APEX. These pumps connect to a carrier solution and the center of a selection valve. The pumps have two types of holding coils associated with them: a circular coil to provide mixing by the "racetrack" effect and a switchback coil that alternates between streamlines that undergo the racetrack effect. Both types of holding coils provide utility for evaluation purposes.

Three 10-port selection valves are also part of the Fluidic Sub-System to provide flexibility and to isolate the sample prep from the PCR/APEX fluidics. One of the selection valves serves to aliquot sample preparation reagents to the cartridge with an embedded Akonni TruTip, another serves to aliquot PCR and APEX pellet rehydration buffers to the cartridge, and the third serves as a means of venting or applying positive pressure to reservoirs on the cartridge. Increasing the number of ports per selection valve is one way to increase the number of cartridges per instrument without adding hardware complexity.

The cartridge pin valves serve two purposes: (1) they allow a single reagent supply line to serve multiple reservoirs on the cartridge and (2) they control liquid movement that would otherwise be directed in unwanted pathways due to the compliance of air. Miniature linear actuators open and close the cartridge pin valves due to their high force.

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Small holders were designed and implemented to rigidly position the actuators concentric with the shaft of the pin valves. The holder design allows the actuator to be rotated along the axis of the pin valve shaft to tightly pack multiple actuators into place. This design is necessary because the body of the actuator protrudes from the axis, giving it a quasi-elliptical profile (*i.e.*, the actuators are not axisymmetric). The actuators are fastened to an I-Beam to rigidly secure them to the instrument. The arm of the actuator penetrates through a hole on a second I-Beam, which provides support for the cartridge. This second I-Beam has a linear pattern of threaded holes that allow ¼-20 standoffs to be fastened to it. The standoff also has a threaded hole that accepts a bolt, which penetrates through the cartridge. The bolts keep the cartridge in a fixed position with respect to x, y and z dimensions. This assembly design has been found to be successful in implementation. The screws, which are manually threaded to secure the cartridge, will likely be replaced by linear actuators or solenoids that drive multiple locating shafts through the body of the cartridge to secure it in place during a future generation of the instrument.

Bladder Thermal cycler Sub-System: The Bladder Thermal Cycler Sub-System consists of 2 pumps, 3 three-way valves, 3 heaters (two for the denaturing flow loop and one for the annealing/extension flow loop), 2 reservoirs that serve as bubble traps and refilling access, a radiator, and a bladder (Figure 12). A survey of pumps was undertaken to identify pumps that are low-cost (<$300), have ¼" NPT fittings, have a flow rate in the range of 0.1 to 1 LPM, is compatible with oils and coolants, are compact, and can operate at temperatures up to 85°C. The best pump candidate that we identified was a $100 Facet fuel-pump, which is a diaphragm pump. This type of pump has unidirectional flow, which is acceptable for this application. It's compatible with oils and coolants, and has an acceptable flow rate range, and operates up to 82°C. To protect the temperature of the pump from the heaters, a cPVC fitting was used to thermally isolate the heater from the pump. The previously-used three-way Granzow valves also met similar requirements to these, and so were taken forward as part of the design process. The previous design of the heaters consisted of welding two halves of aluminum with parallel serpentine tracks that traversed the aluminum block. We went to a simpler design that makes use of off-the-shelf parts: coiled AC heaters from Watlow tightly couple a thin-walled copper pipe that has ¼ NPT fittings soldered on both ends.

The reservoirs that were selected were stainless steel thermoses because they are off the shelf and have good insulating properties. A radiator was initially used to cool the cold zone loop, but was later determined to be unnecessary. The cold zone becomes hot due to mixing with the hot zone loop when the valves switch state. An off-the-shelf memory cooling device was used for the bladder. This device is 4" long and is well suited to the dimensions of the flow cell.

To implement the low temperature PCR, formamide was added to the PCR reaction to chemically lower denaturing and annealing melting temperatures. The added advantage of this low temperature PCR is that we can implement a wider variety of materials for the thermal cycler and the flow cell attached to the cartridge.

The ramp times with this system are approximately 10°C/sec for both heating and cooling. For preliminary evaluation of performance for this iteration of the Bladder thermocyler, a PCR reaction was performed in a Cepheid Smart Cycler tube (flat reaction tube) and flow cells inserted between the

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bladders in the Bladder Thermal cycler and a standard 0.2 ml PCR tube inserted in an MJ Reseach thermal cycler. The product gel in Figure 12b shows that both thermal cyclers generated equivalent levels of product. This is the result of the implementation of computer and pump feedback control of the Bladder thermal cycler to eliminate annealing temperature undershoots experienced in Phase I and allow faster thermocycling. Figure 13 demonstrates PCR in a flow cell and subsequent APEX, showing equivalent discrimination in both a tube-based format and a Cepheid Smart Cycler tube on the bladder thermal cycler.

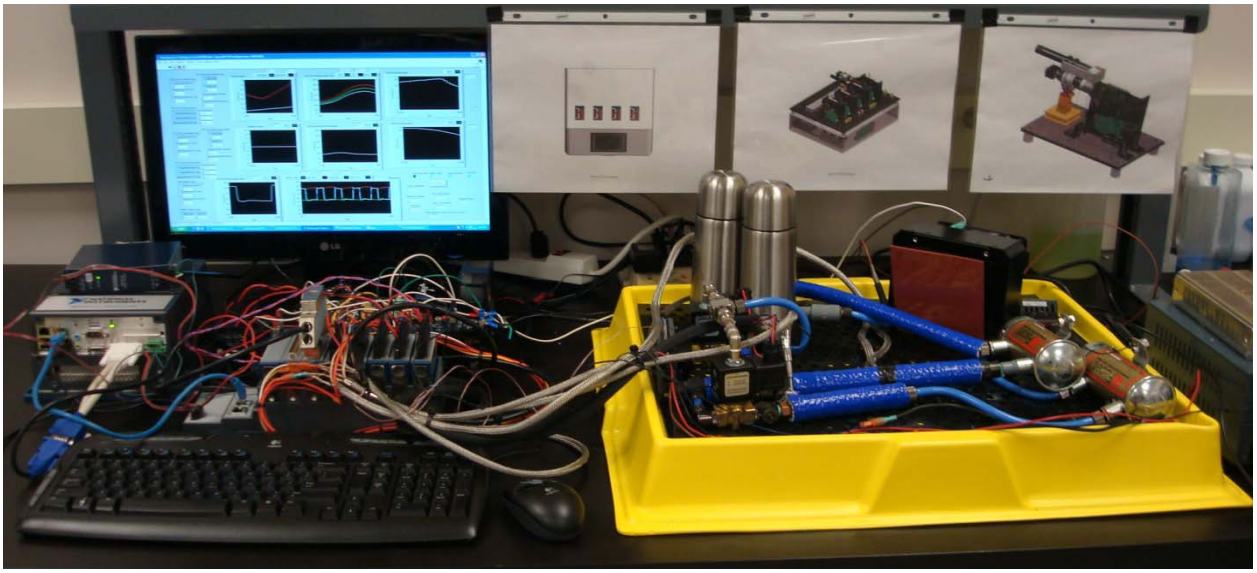

Figure 12a. Stand alone bladder thermal cycler sub-system used for optimization and evaluation testing.

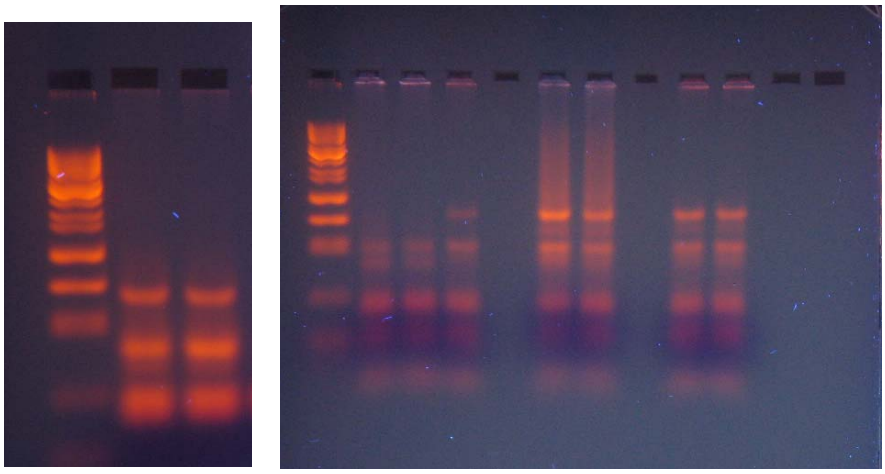

Figure 12b. (left) A multiplex amplification reaction carried out on an MJ thermal cycler (lane 2) and in an Cepheid Smart Cycler tube inserted in the Akonni Bladder thermal cycler (lane 3). The upper two bands, consisting of 6 PCR amplicons, are template for the subsequent APEX reaction. Lane 1 is the marker lane. (right) A multiplex amplification carried out on the bladder thermal cycler using flow cells. Lanes 1 and 2 are experiments done on the flow cell and bladder thermal cycler, lane 3 is an experiment done on the Cepheid tube on the bladder thermal cycler, lanes 5 and 6 are flow cells amplfied on a quanta thermal cycler, and lanes 8 and 9 are amplifications performed in tubes on an MJ thermal cycler.

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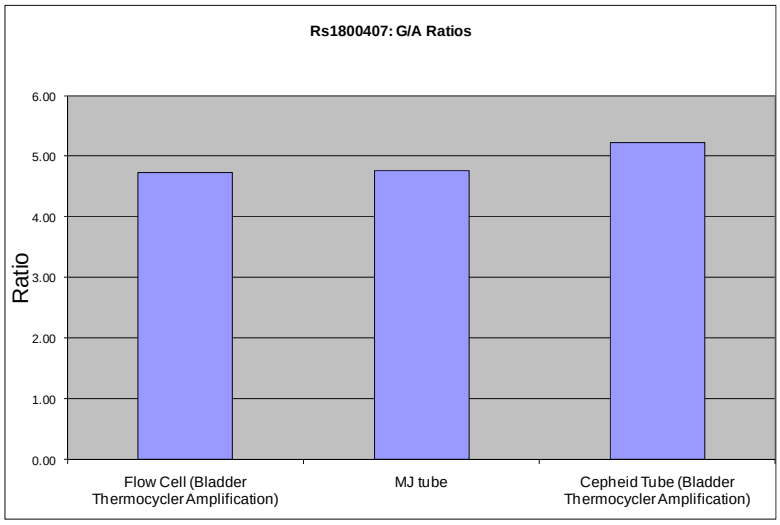

Figure 13. (left) Flow cell. PCR chamber on the bottom and APEX chamber on the top. (right) APEX allele signal ratio results obtained for an eye color SNP at position RS1800407. PCR was performed in the flow cell positioned in the bladder thermal cycler. Flow cell results were compared to a positive control (0.2 ml PCR tube) amplified in an MJ thermal cylcer and a Cepheid tube also positioned in the bladder thermal cycler. APEX was performed offline for one hour. Results indicate comparable APEX signals for all three PCR approaches and confirm the homozygous GG genotype.

Optical Sub-System: The Optical Sub-System, developed for acquiring fluorescence patterns associated with the test-specific interaction of the APEX generated labeled products associated with oligonucleotide primers immobilized in the gel elements of array, is built around a high-quality off-the-shelf imaging optics, a compact low-noise monochrome 1/3" CCD camera, and a high-intensity LED as a fluorescence excitation source.

The optical design was facilitated by the long working distance (39 mm) and a relatively high light collecting efficiency (NA = 0.234) of the Planapo 2x objective lens. Since the objective is infinity-corrected, the array surface of the slide should be positioned at the front focal plane of the lens. The emission filter is located in the infinity space between the objective and video lens and two-component beam expander comprising a plano-concave lens and an achromatic doublet. The beam expander (not shown) reduces the magnification factor of the entire lens system to 0.75x. With the current CCD sensor having 1/3" format and a 7.4 µm pixel size, this magnification adjustment allows imaging arrays of up to 12x18 gel elements with a spatial resolution (limited by the CCD array pixel size) of about 10 µm.

The fluorescence excitation channel implements the Köhler illumination scheme for a projection system, which ensures uniform (within 3%) illumination of the object plane despite the complex structure of light emitting region of the LED. The bandpass clean-up filter placed between the collector and condenser lenses cuts off the long-wavelength wing of the LED emission spectrum that overlaps with the fluorescence band of Cy3.

Figure 14 shows the imaging system fully assembled and mounted on a chassis used for its testing and making adjustments to the beam incidence angle using the beam-steering mirror.

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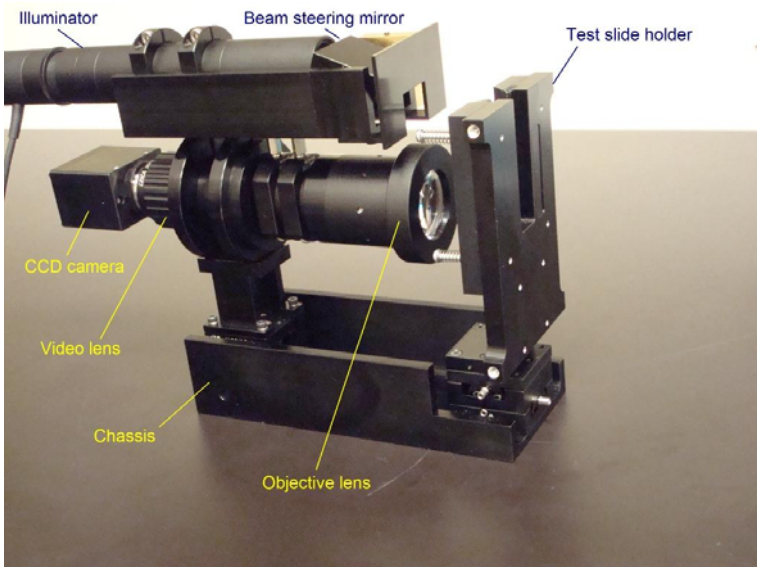

Figure 14. Imaging system fully assembled and prepared for testing.

III. RESULTS

In Phase I, we adopted Y-chromosome SNP markers as a model system. Phase I (Award number 2007-DN-BX-K145, "A Low Cost Microfluidic System for Typing Y Chromosome SNPs"; awarded on September 11, 2007, end date: February 28, 2009) demonstrated the feasibility of implementing advanced TruArray components and assays for forensic typing of SNP markers. However, while Y-chromosome SNPs have forensic utility, Y-chromosome STRs are more useful and practical. However, SNPs are clearly much better suited to implement into the emerging physical appearance determination application [1]. Elucidating an individual's appearance (*e.g.*, eye color, hair color, height, etc.) from a forensic sample can provide critical information to identify a suspect (especially if an STR profile is not in the CODIS database) or a victim (*e.g.*, samples could include blood, highly degraded remains, body parts, or other trace evidence).

The purpose and scope of our efforts, therefore, transitioned to the development of a microfluidic-controlled system for SNP-typing of physical appearance markers. Phase II was to improve, refine, integrate and automate components or subcircuits developed in Phase I.

ASSAY DEVELOPMENT

The path forward for an eye color assay demonstration picked up on assay formulations developed for Y-chromosome SNPs in Phase I. Sequence information for the SNPs was compiled and forward and reverse PCR primers were synthesized. Single-plex PCR reactions for each amplicon were tested and optimized. Systematic pooling of the primers in a multiplex format to produce a standard 6-plex PCR reaction for the eye color amplicons was achieved. This standard 6-plex PCR reaction was converted to

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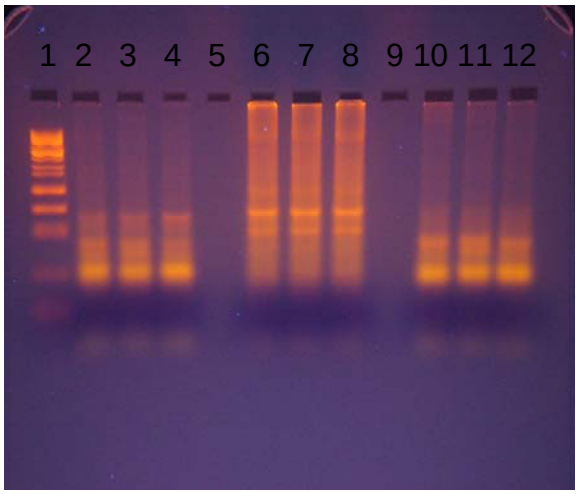
a low temperature (LowTemp) 6-plex PCR reaction. LowTemp PCR involves using a chemical to supplement temperature annealing stringency. By adding formamide (a common PCR additive) to the master mix, the denaturing temperature was reduced to 85°C instead of the normal 95°C. This strategy allows more choices of plastics and adhesives for our integrated cartridge; thereby lowering manufacturing costs. In addition, the lower temperature allows more material choices for the bladder thermal cycler components and plumbing.

Establishing Baseline PCR and APEX Conditions: Multiplex PCRs were performed using Qiagen Taq polymerase. The initial denaturation temperature was set for 3 min at 85°C, followed by 2-temperature thermal cycling at 85 °C for 15 sec and 56, 56.9, or 58.0°C. Successful generation of PCR products was observed on agarose gels (Figure 15 lanes 2-4). Other enzymes tested for multiples PCR were Deep Vent Exo Taq polymerase and TaKaRa Exo Taq polymerase, since these enzymes can be used for APEX reactions. The results showed that Deep Vent Exo Taq polymerase (Figure 15, lanes 6-8) and TaKaRa Exo Taq polymerase (Figure 15, lanes 10-12) yielded inferior product gel profile compared to that of Qiagen Taq polymerase. However, TaKaRa Exo Taq polymerase performed better than Deep Vent Exo Taq polymerase, and therefore could potentially be optimized as a single enzyme approach for both PCR and APEX.



Low-temp (2.5% formamide):
3 min at 85oC;
45 cycles: 85oC – 15s; 56-58oC – 60s;
3 min at 58oC;
Hold at 4oC

Lane	Annealing temperature; Polymerase	Lane	Annealing temperature; Polymerase
1	50-bp Marker	7	56.9°C; Deep Vent Exo-/Deep Vent Exo- Buffer
2	56.0°C; Qiagen Taq/Qiagen Multiplex Mix	8	58.0°C; Deep Vent Exo-/Deep Vent Exo- Buffer
3	56.9°C; Qiagen Taq/Qiagen Multiplex Mix	9	
4	58.0°C; Qiagen Taq/Qiagen Multiplex Mix	10	56.0°C; TaKaRa Exo/TaKaRa Exo Buffer
5		11	56.9°C; TaKaRa Exo/TaKaRa Exo Buffer
6	56.0°C; Deep Vent Exo-/Deep Vent Exo- Buffer	12	58.0°C; TaKaRa Exo/TaKaRa Exo Buffer

Figure 15. Agarose gel of products generated from LowTemp 6-plex PCR for eye color amplicons.

The agarose gel was useful for a general indicator of PCR multiplex performance. Since some amplicons were similar in size, a more informative test was required. Therefore, the Akonni gel drop hybridization microarray was used to detect the presence of each amplicon generated in the PCR reaction. This was accomplished by immobilizing one primer for each of the six amplicons onto the array. These immobilized primers served as hybridization probes for PCR amplicons that were generated using Cy3 5'-end-labeled primers.

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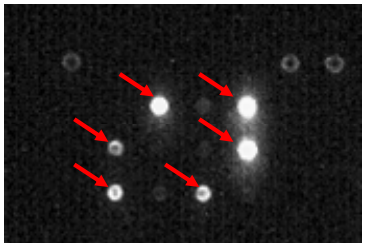
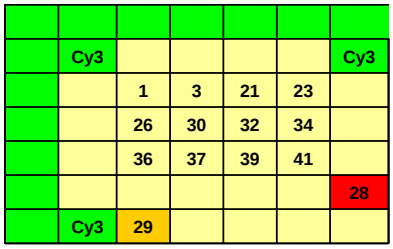
LowTemp 6-plex PCR product generated using the Qiagen Taq polymerase at three different PCR annealing temperatures was subjected to microarray hybridization analysis. Figure 16 displays a microarray image and fluorescent intensities of gel spot signals for each amplicon generated in the reaction. All six amplicons were detected, with amplicon signal intensities exceeding the acceptable threshold for a positive hit on the array. Relative signal intensities amongst the products did vary, but this may be consequential of the array hybridization step and not the PCR step. Nevertheless, this was further characterized as the standard hybridization array was replaced with APEX. Signal intensities can be fine tuned by adjusting primer concentrations in the multiplex PCR and/or length of primers used for APEX.





FULL Low-Temp PCR; 2.5% Formamide; Qiagen Taq;				
Probe#	ID	56.0oC; FU	56.9oC; FU	58.0oC; FU
3	A-Rs1393350_Up_G19	211605	133430	153239
23	A-Rs16891982_Up_C20	329354	452999	327053
26	A-Rs1800407_Up_A17	6464	51617	21439
34	A-rs12913832_Dn_C20	126539	174361	234627
36	A-rs12896399_Up_T23	20781	42752	31572
39	A_rs12203592_Up_C21	8513	12270	29532

Figure 16. Microarray results for 6-plex PCR products for six different loci. (Upper left) microarray image with arrows pointing to the six PCR product generated in the multiplex PCR, (upper right) microarray map, (lower) probe number/primer identifier/fluorescent signal intensities for PCR product generated under three different annealing temperature.

Table 6. Predicting eye color phenotypes by AS-APEX genotyping

Rank	SNP ID	Common Allele	Minor Allele	Sample Number				
				NPS24	NPS25	NPS28	NPS29	NPS30
1	rs12913832	G / Blue	A / Brown	GG	GA	GG	GA	GG
2	rs1800407	C / Brown	T / Blue	CC	CC	CC	CC	CC
3	rs12896399	T / Blue	G / Brown	GG	GG	GG	GG	GG
4	rs16891982	G / Blue	C / Brown	GC	GC	GC	GG	GG
5	rs1393350	G / Brown	A / Blue	GG	GG	GG	GA	GA
6	rs12203592	C / Brown	T / Blue	CC	CC	TT	CC	TT
predicted phenotype				blue	brown	blue	brown	blue
actual phenotype				blue	brown	blue	brown	blue

In the Akonni APEX approach, primers are designed so that a single primer is immobilized within each gel element on the array 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. In the presence of the correct target and matched 3’ base, polymerase incorporates fluorescently-labeled nucleotides to produce the final signal. In Phase II, we streamlined the assay to isothermal conditions for eye color SNPs. The six SNP loci listed in Table 1 were typed by our AS-APEX assay on samples provided by the Massachusetts State Police Forensic Services Group. As summarized in Table 2, the AS-APEX results show good

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correlation of the determined genotypes and predictive phenotypes with the individual's actual phenotype. Each genotype in Table 2 is shaded either brown or blue to indicate the potential influence on eye color phenotype (also shaded accordingly). The SNPs are ranked by level of importance in influencing eye color. For example, individuals that are homozygous GG at position rs12913832 have a 99% probability of exhibiting blue eyes regardless of the genotypes at the other loci.

The feasibility and functionality of the assay on Akonni TruTip purified samples was verified by using 8 ng of DNA isolated from blood (Figures 17) and semen (Figures 18). Correctness of genotype calling was confirmed by sequencing.

Blood DNA Ratios					Results
Sample ID	Description	Average	Average	Grand Average	
50	Rs1393350: G/A	0.78	0.83	0.80	A/G
53	Rs1393350: A/G	1.29	1.21	1.25	
23	Rs16891982: C/G	5.75	3.98	4.68	C
24	Rs16891982: G/C	0.17	0.25	0.21	
57	Rs1800407: G/A	2.18	1.47	1.77	G
60	Rs1800407: A/G	0.46	0.68	0.56	
33	Rs12913832: A/G	4.03	3.61	3.80	A
34	Rs12913832: G/A	0.25	0.28	0.26	
66	Rs12896399: G/T	0.18	0.19	0.19	T
69	Rs12896399: T/G	5.50	5.18	5.31	
71	Rs12203592: C/T	11.31	9.13	10.08	C
73	Rs12203592:T/C	0.09	0.11	0.10	

Figure 17. Primer (allele) signal ratio values for each SNP to deduce genotype of the blood sample. Duplicate average ratios of probes for the each mutation are reported as well as the average of these two. The called genotype matched the genomic DNA sequencing results.

Semen DNA Ratios					Results
Sample ID	Description	Average	Average	Grand Average	
50	Rs1393350: G/A	0.21	0.29	0.25	A
53	Rs1393350: A/G	4.75	3.42	3.94	
23	Rs16891982: C/G	0.04	0.03	0.03	C
24	Rs16891982: G/C	25.51	35.75	30.24	
57	Rs1800407: G/A	1.59	1.60	1.59	G
60	Rs1800407: A/G	0.63	0.63	0.63	
33	Rs12913832: A/G	1.10	1.02	1.06	A/G
34	Rs12913832: G/A	0.91	0.98	0.95	
66	Rs12896399: G/T	0.17	0.16	0.16	T
69	Rs12896399: T/G	5.95	6.26	6.09	
71	Rs12203592: C/T	1.14	1.33	1.23	C/T
73	Rs12203592:T/C	0.88	0.75	0.81	

Figure 18. Primer (allele) signal ratio values for each SNP to deduce genotype of the semen sample. Duplicate average ratios of probes for each mutation are reported as well as the average of these two. The called genotype matched the genomic DNA sequencing results.

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From the baseline assay performance and communications with the assay lyophilization experts (BioLyoph), we recognized the need to: 1) modify PCR and APEX conditions to complete the assay by 2 hours, 2) redesign APEX primer pair for SNP rs1800407 since correct typing was achieved only at elevated temperatures (67.5 – 70°C), at which signals from some of the other SNP primer pairs became weaker, and 3) modify PCR and APEX conditions to decrease glycerol concentrations to meet Biolyph requirement for lyophilization.

Modification of Conditions to Reduce Assay Time: After reviewing all the gel data obtained under varying cycling numbers and anneal/extend step times, 1 hr 35 min on the MJR was the fastest time that generated an acceptable product yield. Figure 19 is an example of one of the gels, showing results using the MJR and Piko thermal cyclers set at 40 cycles with a 60 sec annealing/extension step. Since APEX has been typically performed for 60 min, the current total assay time is about 2 hr 35 min.

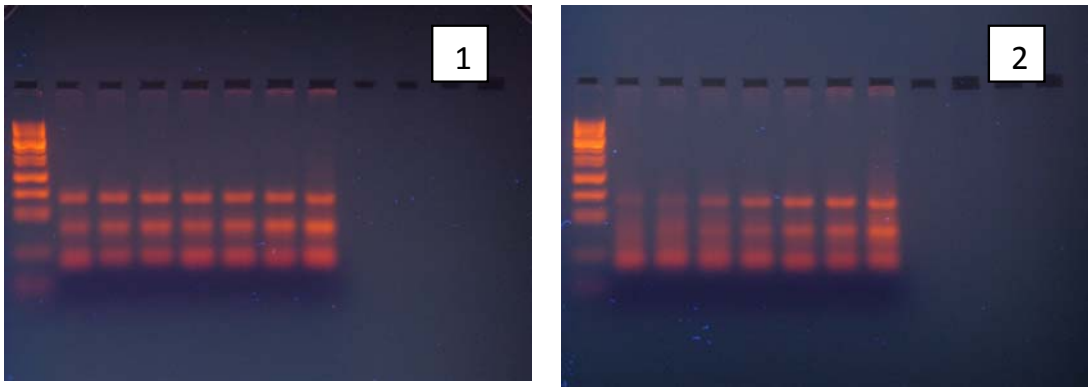

Figure 19. Electrophoresis of multiplex PCR products amplified under different conditions. DNA isolated from blood using TruTip and taken in different amounts for PCR. For each gel, lanes left to right: 1. Marker; 2 DNA, 1 ng; 3. DNA; 2 ng; 4. DNA, 5 ng; 5. DNA 10 ng, 6. DNA, 20 ng, 7. DNA, 50 ng, 8. DNA, 100 ng.
(Gel 1) **MJR thermal cycler**: 86.5^{o}C-3 min; [86.5-15s; 56°C-1 min; 40 cycles]; Total time 1 h 35 min.
(Gel 2) **Piko thermal cycler**: 86.5^{o}C-3 min; [86.5-15s; 56°C-60s; 40 cycles]; Total time 1h 15 min.

Redesign APEX primer pair for SNP rs1800407: APEX probe functionality was tested using mixes of synthetic oligonucleotides templates mimicking mixes of single-stranded amplicons with all possible SNPs of interest. Figure 20 shows some examples of this testing. All SNPs were detected correctly. As expected, the rs1800407 pair, while giving correct detection showed rather low discrimination ratio (1.5-2.5). In order to enhance the ratio, new additional rs1800407 probes were redesigned and a new APEX microarray was synthesized and tested as shown in Figure 21. The array was challenged with 10ng of purified DNA using TruTip extraction.

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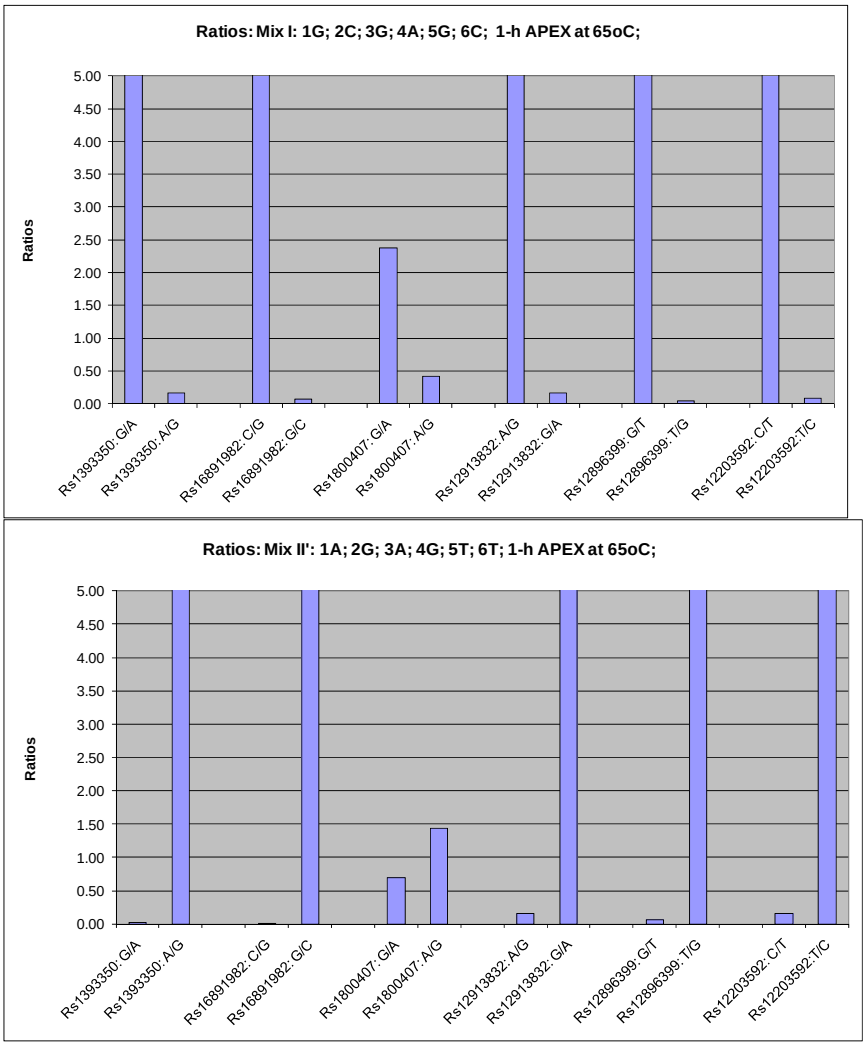

Figure 20. Primer (allele) signal ratio values for each SNP correlates to the synthetic template mixtures. All primer pairs work well except for Rs1800407. Shown are results from two different mix preparations to represent different genotypes.

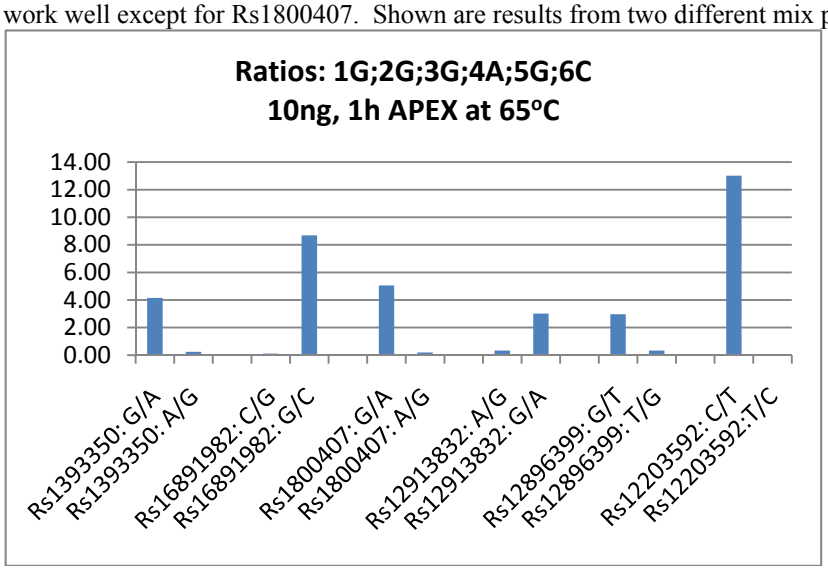

Figure 21. Optimized probe design for Rs1800407. These probes show robust discrimination across multiple samples and tests.

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Lyophilization of PCR and APEX Chemistries: To prepare the PCR and APEX master mixes for lyophilization, the glycerol content of the assay components needed to be determined. Low glycerol content of the assay chemistry is crucial for proper lyophilization. This required gaining as much information as available about the commercial enzymes and buffer recipes that were being incorporated in the reaction recipes. Biolyph was responsible for the lyophilization of the reagents. As shown in Figure 22, lyophilization of both PCR and APEX mixes with different excipient concentrations at Biolyph was successful. The report from Biolyph stated:

"We have completed the lyophilization of your six (6) reagent variations from MPI #AKB-071510. We have inspected their quality following lyophilization and prior to their packaging. The PCR-2S, PCR-4S, and PCR-7S all yielded the same quality. These were impacted by the effect of glycerol, which lowered their freezing point, and created what we call a "brain-head" texture to the spheres, which demonstrates that the structure collapsed to a small extent. These also demonstrated fragility in a frozen state. The APEX-2S, APEX-4S, and APEX-7S all yielded beautiful LyoSpheres™."

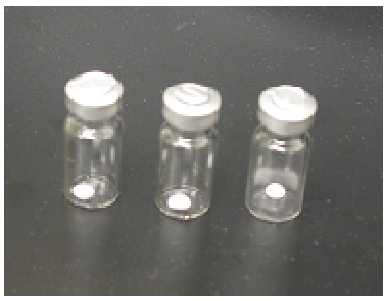

Figure 22. Lyospheres containing PCR and APEX master mixes.

Test results resulted in good or acceptable performance for lyophilized Taq Polymerase, lyophilized Deep Vent Exo-, and lyophilized full APEX reaction mix (Figure 23). However, lyophilized PCR buffers showed weaker amplification efficiencies in comparison to freshly prepared PCR buffers. Only single-plex PCR could be successfully executed with lyophilized PCR buffer and even for single-plex PCR, the product was noticeably less efficient in comparison with fresh PCR buffer (Figure 23).

Given the weak multiplex PCR results using the Lyospheres, We proceeded with single-plex PCR for PCR and APEX Lysopheres testing. Table 7 contains APEX fluorescent signals obtained for rs1800407 detection using different combination of fresh and lyophilized components. These ranged from complete fresh reaction mixes used in PCR and APEX to complete lyophilized mixes used for PCR and APEX. Even using the complete set (Mix 5) of PCR and AS-APEX Lyospheres, the correct eye color genotype of GG was obtained.

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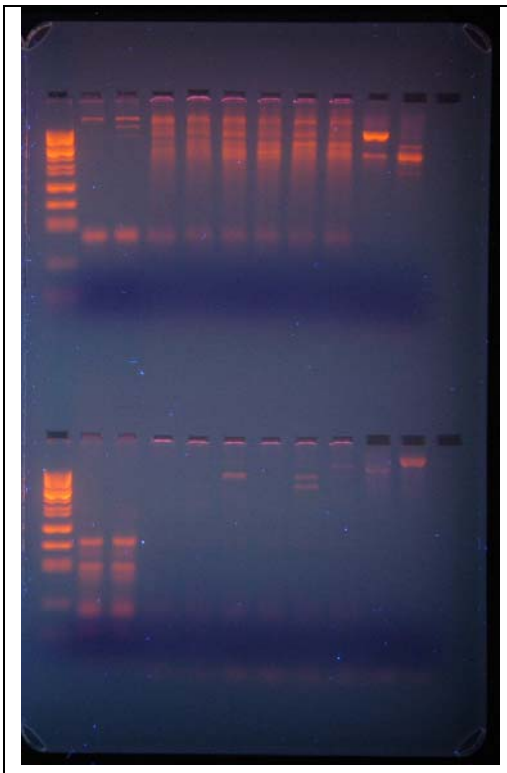

Upper Part rs1800407 Single-plex PCR
1 50-bp marker
2. Fresh Qiagen Multiplex Mix + RS1800407 primer pair + Fresh Taq
3. Fresh Qiagen Multiplex Mix + RS1800407 primer pair + Fresh Taq
4. Lyophilized Qiagen Multiplex PCR Mix-2S+RS1800407 primer pair +2xTaq LS 2S
5. Lyophilized Qiagen Multiplex PCR Mix-2S+RS1800407 primer pair +2xTaq LS 2S
6. Lyophilized Qiagen Multiplex PCR Mix-4S+RS1800407 primer pair +2xTaq LS 2S
7. Lyophilized Qiagen Multiplex PCR Mix-4S+RS1800407 primer pair +2xTaq LS 2S
8. Lyophilized Qiagen Multiplex PCR Mix-7S+RS1800407 primer pair +2xTaq LS 2S
9. Lyophilized Qiagen Multiplex PCR Mix-7S+RS1800407 primer pair +2xTaq LS 2S
10. Lyophilized Qiagen PCR Buffer Mix-4s+RS1800407 primer pair+2xTaq LS 2S
11. Lyophilized Qiagen PCR Buffer Mix-4s+RS1800407 primer pair+2xTaq LS 2S
Lower Part: Multiplex PCR
1. 50-bp marker
2. Fresh Qiagen Multiplex PCR Mix+ Fresh Taq
3. Fresh Qiagen Multiplex PCR Mix+ Fresh Taq
4. Lyophilized Qiagen Multiplex PCR Mix-2S+2xTaq LS 2S
5. Lyophilized Qiagen Multiplex PCR Mix-2S+2xTaq LS 2S
6. Lyophilized Qiagen Multiplex PCR Mix-4S+2xTaq LS 2S
7. Lyophilized Qiagen Multiplex PCR Mix-4S+2xTaq LS 2S
8. Lyophilized Qiagen Multiplex PCR Mix-7S+2xTaq LS 2S
9. Lyophilized Qiagen Multiplex PCR Mix-7S+2xTaq LS 2S
10. Lyophilized Qiagen PCR Buffer Mix-4s+2xTaq LS 2S
11. Lyophilized Qiagen PCR Buffer Mix-4s+2xTaq LS 2S

Figure 23. PCR product gel using Low-temp single-plex for rs1800407 (upper part) and multiplex amplification (lower part) for with fresh and lyophilized reaction components.

Table 7. APEX signals for each allele obtained using different mixes of fresh and lyophilized (LS) reaction components to type the eye color rs1800407 marker.

#	Description	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5
77	A-Rs1800407_Up _G15_NW3	630613	116906	52511	47681	48643
78	A-Rs1800407_Up _A15_NW3	173880	23366	15651	11410	15208

Mix 1: Fresh Qiagen Multiplex PCR Buffer; Fresh Taq Polymerase; Fresh APEX Buffer; Fresh Deep Vent Exo-
Mix 2: LS Qiagen Multiplex PCR Buffer; 2xLS Taq Polymerase; Fresh APEX Buffer; Fresh Deep Vent Exo-
Mix 3: LS Qiagen Multiplex PCR Buffer; 2xLS Taq Polymerase; LS APEX Buffer 2S; LS Deep Vent Exo- 2S
Mix 4: LS Qiagen Multiplex PCR Buffer; 2xLS Taq Polymerase; LS APEX Buffer 4S; LS Deep Vent Exo- 2S
Mix 5: LS Qiagen Multiplex PCR Buffer; 2xLS Taq Polymerase; LS APEX Buffer 7S; LS Deep Vent Exo- 2S

Multiplex testing for APEX using the APEX Lyospheres was evaluated using fresh multiplex PCR mixes. Figure 24 shows that correct genotype were called for 4 of the 6 SNPs. One additional SNP would have been called correctly but with low confidence, and the remaining SNP would have been incorrectly called as heterozygous at that position. The indeterminate and missed call was due to low raw signals generated. Nevertheless, the results are Akonni's first demonstration of feasibility of a custom APEX lyophilized pellet. Switching to a hot start Taq polymerase for the multiplex PCR and

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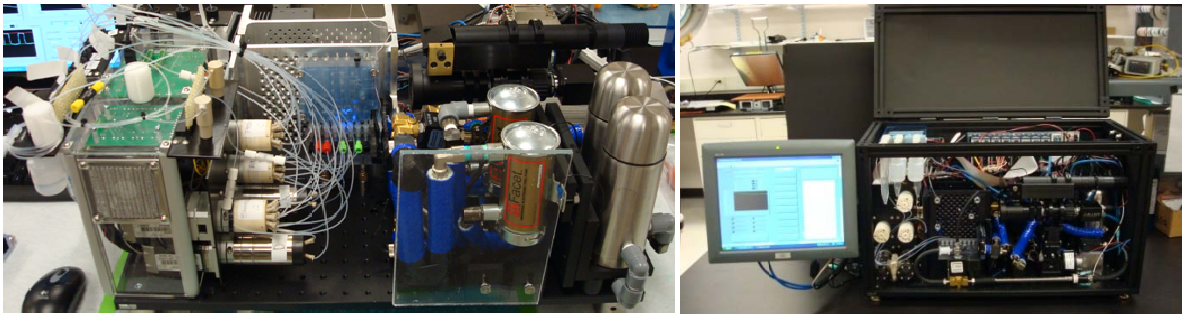
further optimization of formulations to increase signal intensities could increase signal intensities to correctly type all 6 SNPs. PCR Lyospheres, consisting of the complete buffers system, were lyophilized without the primers to allow testing across multiple primer sets. Primer lyophilization is routinely performed, and is not expected to present technical difficulties.

Blood DNA Ratios					Results
Sample ID	Description	Average	Average	Grand Average	
50	Rs1393350: G/A	0.77	1.75	1.26	A/G
53	Rs1393350: A/G	1.30	0.57	0.94	
23	Rs16891982: C/G	4.53	8.64	6.59	C
24	Rs16891982: G/C	0.22	0.12	0.17	
77	Rs1800407: G/A	2.46	2.64	2.55	G
78	Rs1800407: A/G	0.41	0.38	0.39	
33	Rs12913832: A/G	2.87	1.39	2.13	A?
34	Rs12913832: G/A	0.35	0.72	0.53	
66	Rs12896399: G/T	0.37	2.67	1.52	T/G?
69	Rs12896399: T/G	2.73	0.37	1.55	
71	Rs12203592: C/T	6.03	59.16	32.59	C
73	Rs12203592:T/C	0.17	0.02	0.09	

Figure 24. Results generated using stable Lyospheres containing all components the APEX formulation. Lyospheres are stored at room temperature. Top - Raw APEX signals for each primer (allele) pair for each SNP. Bottom - Primer (allele) signal ratio values for each SNP to deduce genotype of the blood sample. The called genotype matched the genomic DNA sequencing results.

HARDWARE DEVELOPMENT

Three sub-systems (Fluidic, Thermal cycler and Optical) were integrated onto a single breadboard (Figure 25). The breadboard base allows flexibility to arrange the various components of the system. Additionally, a vertical support structure for the cartridge and the linear actuators has a pattern of holes that allow the valves to be re-positioned as the cartridge design changes.



System 201 **System 202**

Figure 25: Integrated cartridge breadboard with fluidic sub-system on the left side of each system, thermal cycler on the right side of each system, and optical sub-system on the right of each system. The integrated cartridge is shown on system 201. A touch panel display is shown for System 202.

A substantial part of the effort was devoted to: 1) upgrading components to iterations that were manufacturable and could be packaged into a smaller instrument footprint (current instrument size is approximately two cu.ft.), 2) writing and testing software for subsystem control and communication, 3) performing integrated system testing and troubleshooting.

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Code was written in Labview on a National Instruments industrial computer (NI 3110) to control the three major sub-systems (Fluidic Handling Sub-System, Thermal cycler Sub-System, and Optical Sub-System). The NI 3110 has a dual-core processor where one core executes tasks for Windows and the other core executes tasks for a Real-Time operating system (OS). This architecture (Figures 26-28) allows for the execution of high level Windows OS tasks such as managing the user-interface, serial communication and image processing, as well as low-level Real-time OS deterministic tasks such as control of the heaters, linear actuators, thermal cycler pumps, and precisely timed events. The Real-Time OS communicates with the Ethercat I/O module, which scans analog input, analog output, and digital I/O modules.

Each of the three major sub-systems utilized resources from both the Windows and Real-Time OS and require communication between the two operating systems. Within the Windows environment a sequence of tasks, created by the user, is managed and communicated to the appropriate process on either the Real-Time OS or the Windows OS via shared variables. Tasks associated with the Fluidic Handling Sub-System include: change position of the selection valve(s), close/open cartridge valve(s), and dispense/aspirate with microfluidic pumps. Tasks associated with the Thermal cycler Sub-System include: warm up the themocycler and initiate thermocycling. And tasks associated with the Optical Sub-System include: initiate APEX heating and acquire image. Sequences can be saved and imported into the DX3000 Automated Task Execution Program.

The components, classified as being controlled by the Fluidic Handling Sub-System, include: 2 bi-directional pumps, 3 selection valves, and 8 linear actuators, which open and close the cartridge valves. The bi-directional pumps and the selection valves are controlled by a USB serial port. Serial commands are communicated to them via Labview drivers, developed by Global FIA. The control commands for the pump are direction, flow rate, volume and address, and the control commands for the selection valve include selection valve port number and address. Whereas these commands are executed entirely within Windows, the linear actuators are controlled primarily from the Real-Time Operating System. Linear actuators are controlled by an H bridge of 4 MOSFETs for each valve. Two digital I/O lines per actuator trigger the actuator to move forward, move backward, or remain at rest. When the actuator is at rest, no power is required. The actuators include an internal potentiometer to provide feedback of the position of the actuator arm. A calibration routine is used to determine the extents to which the actuator arm reaches, which correspond to a closed or an open position for the cartridge valves. A comparison algorithm is used to compare the desired with the actual location of the arm. The appropriate movements are executed to reach the desired location within a pre-determined tolerance window. Following the calibration routine, each, all, or some combination of the cartridge valves can be closed or opened as a task in the sequencer.

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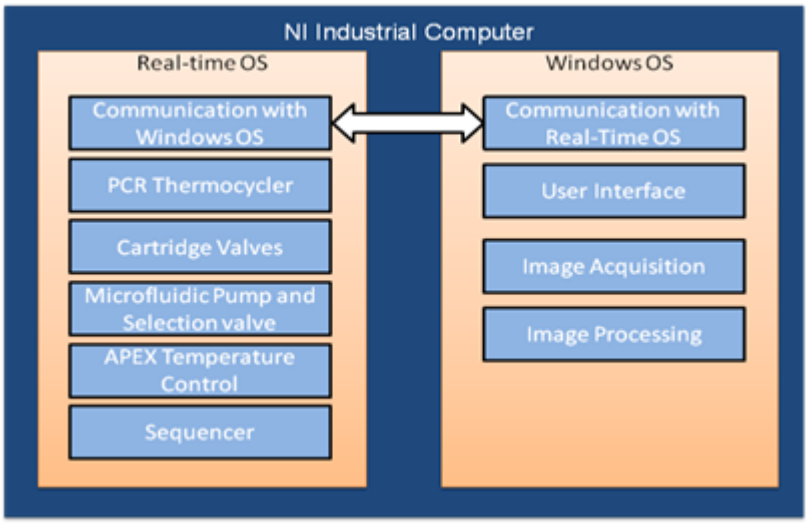

Figure 26. System Architecture Diagram: high-level of processes on both Real-time and Windows OS.

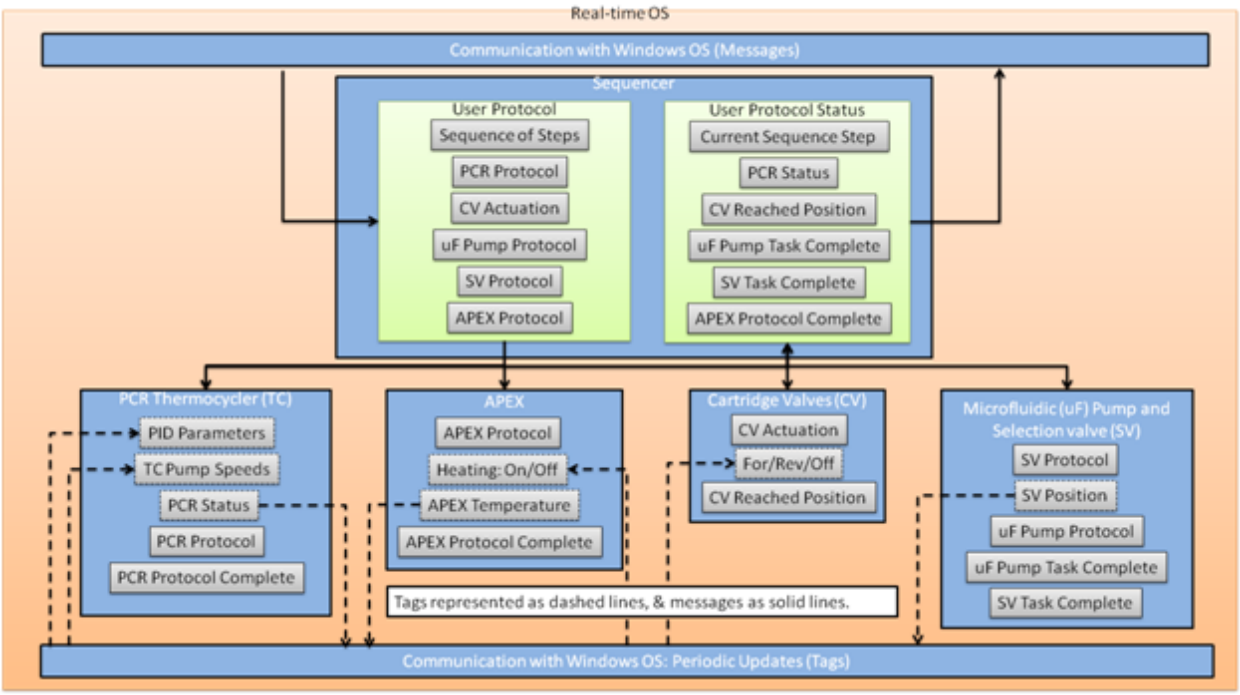

Figure 27. Architecture Diagram: Real-time OS Systems Diagram

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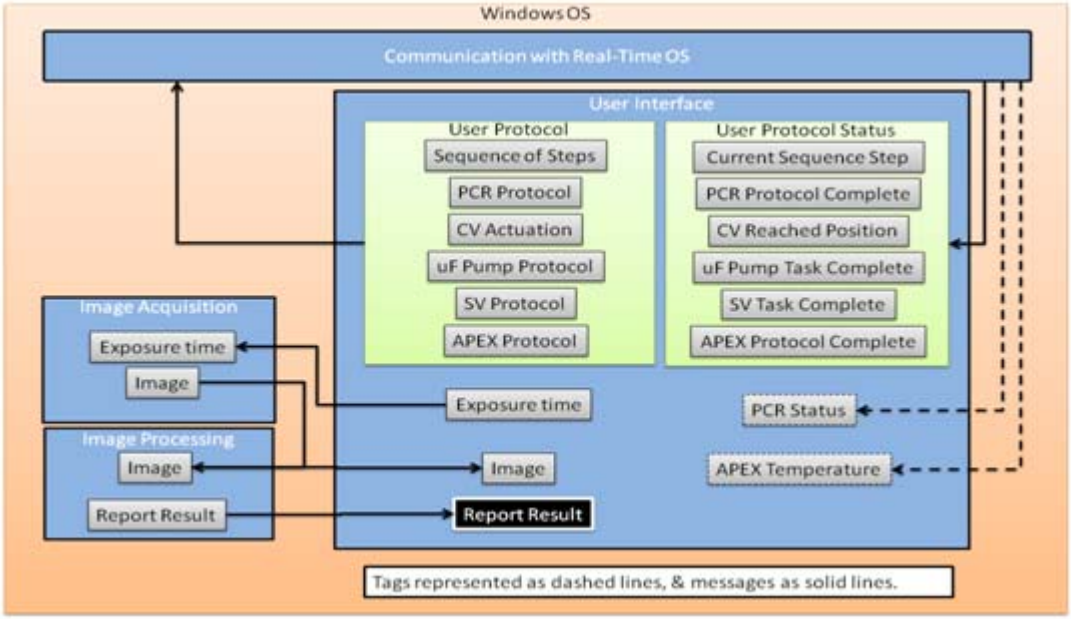

Figure 28. Architecture Diagram: Windows OS Systems Diagram with User Interface

To address the non-uniformity in temperature distributions and slow ramp times associated with commercial slide-based thermal cyclers, we explored an alternative fluidic-based approach. This approach consists of two recirculating fluidic pathways that alternate between a bypass loop and a loop that has a pair of inflatable and compliant "bladders." One pathway is held constant at the high (denaturing) temperature and the other pathway is held constant at the low (annealing and extension) temperature. Before startup when the working fluids are not recirculating, the bladders are relaxed, allowing the flow cell attached to the cartridge to be easily inserted between the bladder pair. During initialization, the recirculating fluids begin to flow and pressurize the "bladder pair", which consequently expand and make tight contact with the flat reaction chambers of the flow cell. During this initialization state, two separate in-line heating units reach the appropriate temperatures (*e.g.*, 95°C and 60°C). Denaturation, which follows the initialization state, is achieved by switching three-way valves, so that the high temperature fluid flows through the "bladder pair" loop, and the low temperature working fluid flows through the bypass loop. During thermal cycling, which follows denaturation, the valves are alternately activated to switch the flow path of the working fluids from this "denaturing" state to an "annealing and extension" state (*i.e.,* the low temperature working fluid flows through the "bladder pair" loop and the high temperature working fluid flows through the bypass loop), and these states continue to switch throughout the thermal cycling protocol.

The components, classified as being controlled by the Thermal cycler Sub-System, include: 2 diaphragm pumps, 3 three-way valves, 3 coiled heaters (two for the hot zone and one for the cold zone), and a fan to provide cooling through a miniature radiator. The radiator was determined not necessary, and therefore is not part of the 202 system. Darlington BJT transistors source current to the diaphragm pumps in proportion to an analog output signal that is controlled by the DX3000 Automated Task Execution Program. Linearly proportional relays control the amplitude of an AC signal that powers the heaters and an AC fan for cooling. Cooling of the recirculating cold zone flow loop helps prevent annealing temperature overshoot due to the mixing of the working fluid from the hot zone and cold

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zone. An analog voltage output signal controls the output of these relays. Three in-line thermistors, exposed to the recirculating flow, are located after the hot zone and cold zone heaters and prior to the bladder. Two separate virtual processes control the recirculating temperature of the hot zone fluidic loop and the cold zone fluidic loop. These virtual processes include an algorithm for PID control of the temperature in the loop. The heater PID is used for a wide range of temperature control, but is slow responding. The pump flow rate PID has a narrow range of temperature control, but is fast responding. So, both PID loops are used to achieve rapid switching between the hot and cold zones with stable plateaus (Figure 29). The radiator fan speed is typically maintained at 40% power.

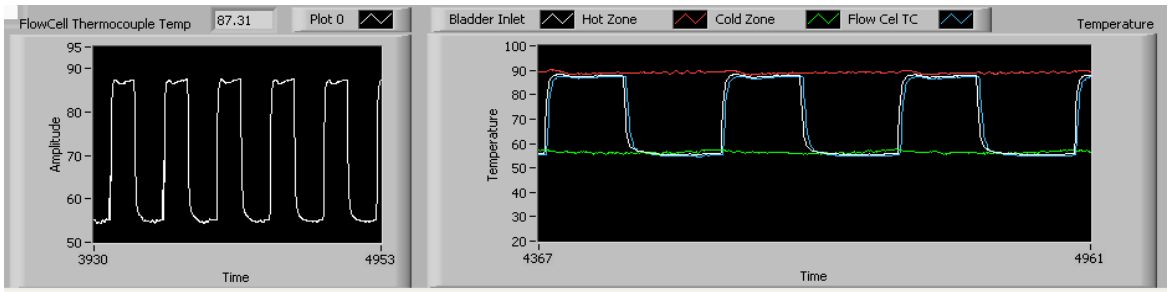

Figure 29. Thermal cycling profile with PID pump and heater control. Red line shows the temperature of the hot zone, green line shows the cold zone, the white line shows the temperature of a thermocouple sandwiched between the bladders, and the blue line shows the temperature of the working fluid just prior to entering the bladder.

The components, classified as being controlled by the Optical Sub-System, include: an LED, a camera, a heater, and an air pump. The LED, camera and air pump are turned on and off by solid state relays. A pulse-width modulation algorithm controls the duty cycle of AC power to the heater using a solid state relay. The air pump flow rate is maintained at a constant rate. A thermocouple, internal to the heater, provides feedback to a PID virtual interface.

We were able to demonstrate automated sample preparation of 50 µL of blood in the cartridge. In this process, all sample and sample waste along with all reagents/buffers that came in contact with the sample were maintained on the cartridge. Used reagents/buffers were moved and stored in the cartridge waste chamber. The entire process was performed under computer control. Real-time PCR results on extracted, purified DNA sample that was manually removed from the cartridge displayed a strong positive PCR signal (Figure 30). More importantly, this purified DNA was successfully used for manual PCR and APEX to call the correct genotype (Figure 31).

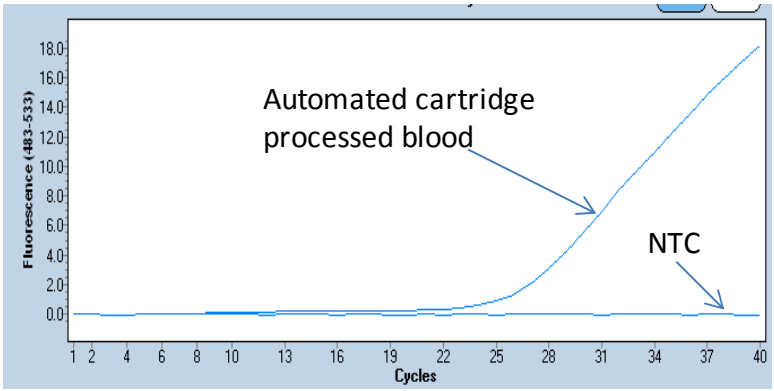

Figure 30. Automated, integrated cartridge-purified DNA was used for manual real-time PCR analysis.

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Blood DNA Ratios					Results
Sample ID	Description	Average	Average	Grand Average	
50	Rs1393350: G/A	0.75	0.77	0.76	A/G
53	Rs1393350: A/G	1.33	1.30	1.32	
23	Rs16891982: C/G	5.33	5.48	5.40	C
24	Rs16891982: G/C	0.19	0.18	0.19	
77	Rs1800407: G/A	5.11	5.13	5.12	G
78	Rs1800407: A/G	0.20	0.20	0.20	
33	Rs12913832: A/G	3.62	3.64	3.63	A
34	Rs12913832: G/A	0.28	0.27	0.28	
66	Rs12896399: G/T	0.34	0.62	0.48	T
69	Rs12896399: T/G	2.91	1.62	2.26	
71	Rs12203592: C/T	31.55	10.83	21.19	C
73	Rs12203592:T/C	0.03	0.09	0.06	

Figure 31. Automated, integrated cartridge-purified DNA was used for (manual PCR (20 ng DNA)/APEX typing. The fluorescent intensities of each primer on the array were imaged, and raw signal intensity data was used to generate primer (allele) signal ratio values for each SNP. The called genotype matched the genomic DNA sequencing results.

Following the successful demonstration of on-cartridge sample preparation, additional studies showed good performance of the on-cartridge PCR using NIST purified DNA and offline APEX. The results were repeatable as shown in Figure 32.

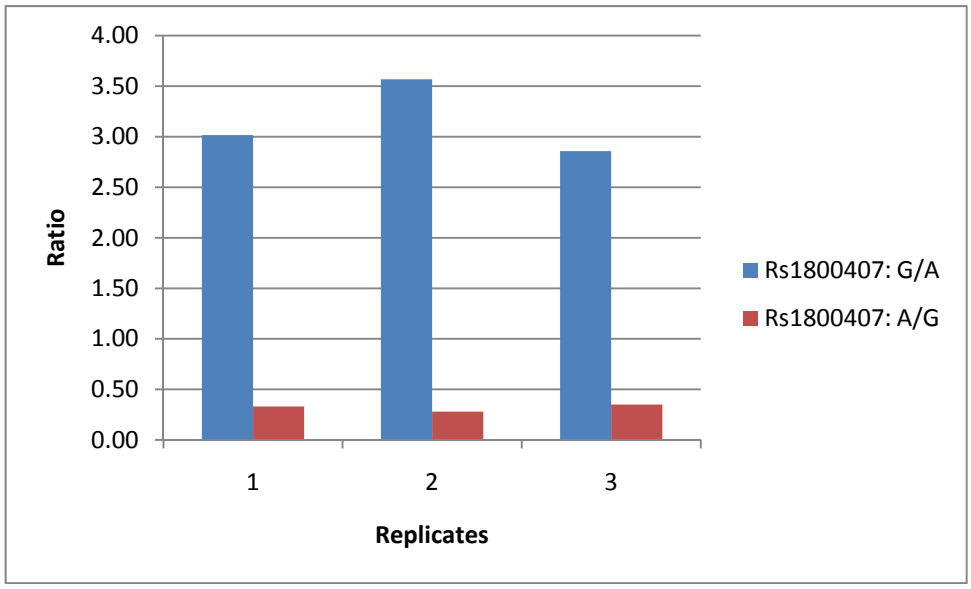

Figure 32. The elution tower was loaded with 10 ng of NIST purified DNA. The microfluidic pump then pushed this solution into the flow cell, which resided in the bladder thermal cycler. The thermal cycler protocol was 3 min – 88°C, and 50 cycles of 88°C for 30 seconds and 53.5°C for 60 seconds, followed by a final step of 53.5°C for 3 minutes. An offline APEX for 1 hour was performed, resulting in the above ratios.

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Complete integration and automation of all the steps was not trivial. A number of technical issues became apparent and required extensive troubleshooting, which did slow the project progress.

****Start of Akonni Proprietary information (in green font) – not for public release****

These technical issues/solutions included:

1. **Reducing PCR inhibition**

We observed that the gasket or spacer tape was inhibiting PCR due to poor adhesion to the film. When carrying out thermal cycling with just water, the water inhibited the PCR reactions, observed as weak bands on a gel. This same pattern was observed for a number of different films, and was more pronounced when there was an obvious compromise with the adhesion to the film. This was the result of poor adhesion and due in part to the unexpected high humidity in the lab (up to 60%) during the summer. We took the necessary measures to control the humidity in the building to improve bonding strengths of the tapes to the films.

2. **Springs on valves**

The o-rings had a high coefficient of friction against the cartridge walls, which required stiff springs to be incorporated into the valve assembly. To compensate for the high spring constant, the highest force Firgelli actuators were included on the system. The tradeoff was speed of actuation. However, since multiple actuators can be controlled simultaneously and since the actuation movement was less than 2 seconds, this seemed like a practical solution.

3. **Bladder o-rings susceptible to silicone oil**

We began experiments with a silicone oil; however, since the off-the-shelf Koolance bladder used silicone o-rings, the seals would fail because of the incompatibility of the two. Silicone oil caused silicone to swell. We therefore switched to propylene gycol, available from Koolance, which has a high heat capacity and good thermal conductivity, but a boiling point of 103. We recently had Koolance make custom bladder assemblies that do not contain silicone o-rings.

4. **Replacement of metal fittings with plastic**

Initial thermal cycling experiments showed slow ramp times when using large metal fluidic connectors (such as brass and steel). So, we custom machined PEEK fittings to replace brass connectors, and we replaced large metal RTDs with small thermistors from Koolance.

5. **Mixing of the hot and cold zone fluids**

When using high flow rates and small reservoir volumes, the heaters did not sufficiently respond to the mixing of the hot and cold zone working fluids after a change in state. So, we increased the volumes of the reservoir (increasing the warm up time), and reduced the flow rates.

6. **Poor signal to noise imaging ratios**

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Although the optical train setup using a separate excitation channel and oblique illumination completely eliminates excitation light scattering inside the objective lens, the LED light that experienced diffuse scattering on both the flow cell and the surfaces of the parts located behind the slide close to it caused elevated background in the array images that reduces the system's dynamic range defined as the difference between the pixel saturation and background levels divided by the standard deviation of the background across the camera frame. Another factor contributing to the overall background level is the fluorescence that the excitation beam may induce in the flow cell spacer and/or cover film. The above mentioned issues were addressed as follows:

- **We reduced the diameter of field diaphragm immediately after the condenser lens. This diaphragm controls the diameter of the beam incident on the slide. Accordingly, reducing the beam diameter to what is actually required for proper illumination of the array can substantially decrease the flux of light that may undergo diffuse scattering or excite unwanted fluorescence.**

- **Introduced a light shield behind the slide. The surface of the shield oriented toward the slide was covered with a black light absorbing material such as self-adhesive flocked paper available from Edmund Optics.**

- **Replaced the Cy3 emission filter with a stack of two similar filters. The Semrock filter used in the current system design has in-band transmission of about 0.95, whereas its transmission within the transmission band of the clean-up filter is of the order of 10^{-6} or even less. However, since in many cases the intensity of scattered LED light is orders of magnitude higher than the fluorescence of gel elements, a small fraction of stray light leaking through the emission filter may bring about a noticeable increase in the image background. Using a filter stack instead of a single emission filter reduces the out-of-band leakage by the factor of 10^{6}. At the same time, the in-band transmission would drop from 0.95 to 0.9 or by only 5%.**

- **Carefully selected a hydrophilic cover film from 3M to have a low intrinsic fluorescence and good optical homogeneity, because under the typical imaging conditions, the film is in close proximity of the gel array, and non-uniformity has a considerable impact on the uniformity of background in array images.**

- **Separation of the PCR flow cell from the array chamber flow cell so that the PCR compatible materials do not need the same properties as those for imaging. The material selection process is still ongoing.**

7. **Due to all the extensive troubleshooting for both assay and hardware development, the goal for the integrated system was reduced to demonstrating sample-to-answer results for one SNP eye color marker. This was achieved at the end of the project, so time ran out to revisit multiplex results and testing on mock forensic samples. However, we were very encouraged to achieve our first automated and integrated results.**

****End of Akonni Proprietary information****

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Despite setbacks, we were successful in demonstrating automated, integrated proof-of-concept results on the 201 system (Figure 33). Starting with a 50 µL sample of blood, automated sample processing, PCR, and APEX was performed for a single eye color maker. The gel spot signal intensities clearly indicated the presence of a C allele and not a T allele at this position. No observable APEX signals for the T allele were detected using the onboard instrument reader, so an offline Aurora imager was used to detect the signal above background for this allele to determine allele ratios. The Aurora imager can achieve this since the imager optics cost an order of magnitude more the Akonni instrument optics. However, our goal was to keep instruments cost low, and further improvements to both assays and hardware efficiencies should allow the detection of these low signals onboard the instrument.

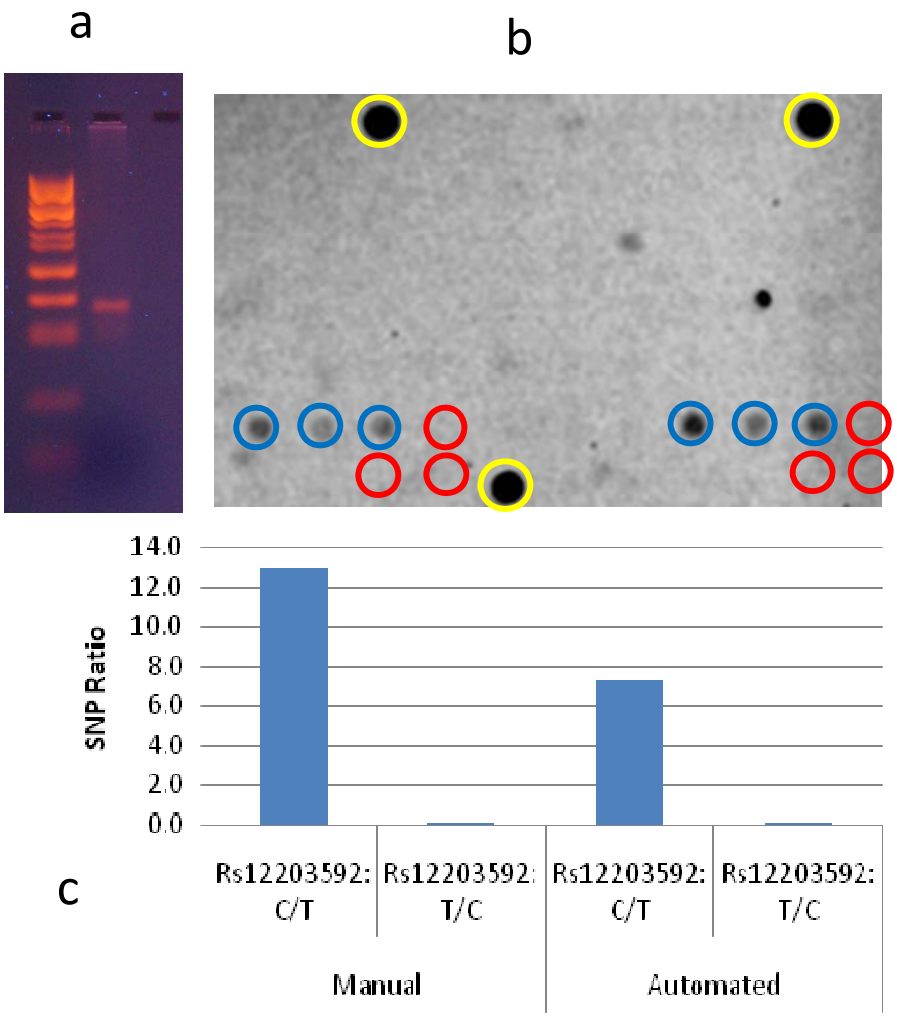

Figure 33. Sample-to-answer results on the integrated system. 50 ul of whole blood was loaded into the cartridge. The integrated system performed automated sample prep, PCR and APEX in the cartridge connected to the instrument. a) Product gel after amplification. b) Image on the 201 system after taking the automated processing through the APEX step. Blue circles denote C allele, red circles denote T allele. c) Allele signal ratios indicating a CC genotype.

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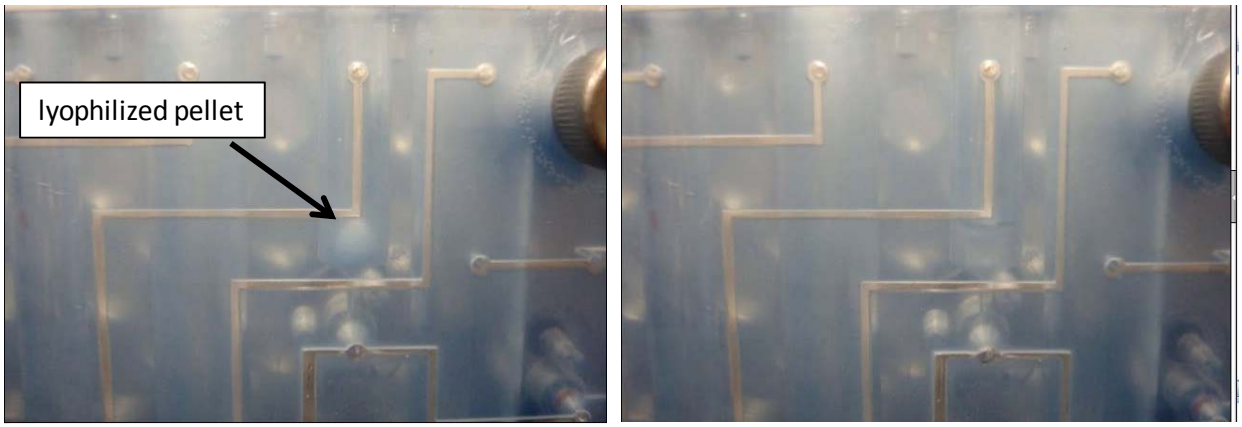
Another functionality demonstrated on the cartridge was the reconstitution of a Lyosphere (Figure 34). The vertical orientation of the cartridge and chamber towers worked well to dissipate trapped air in the Lyosphere. The next step is to implement the Lyospheres into a full sample-to-answer run, but further improvements in assay and hardware development are still required.



before rehydration after rehydration

Figure 34. Automated rehydration of a Lyosphere in the cartridge. The lyophilized pellet was completely dissolved after flowing elution buffer into the elution tower of the cartridge.

IV. CONCLUSION

Discussion of Findings

The main goal of this project was to automate and integrate subcircuits and components demonstrated in Phase I to type SNPs. While STRs are well-suited for making identifications against a reference sample, SNPs are much more useful as genomic markers for the emerging application of phenotype or physical appearance determination. Thus, our ulitimate goal is to produce a small, easy-to-use, rapid, low-cost system that can meet the forensic requirements for this future SNP application.

The goals for this effort were aggressive but realistic, with the main objective to demonstrate automated sample-to-answer results. Here, we summarize the results for each major task, and how well we met the objectives.

Refine allele-specific arrayed primer extension (AS-APEX) protocols and chemistries

Correct SNP identification was demonstrated for multiplex asymmetic PCR and subsequent isothermal APEX on a gel-drop biochip. However, efficient multiplex PCR using the low temp protocol needed better performance. Part of the issue is that the low temp PCR does not utilize hot start Taq polymerase, to avoid subjecting experimental hardware materials to 95°C temperatures. However, recent changes to materials should allow hot start Taq polyerase, and thus improve the mulitplex PCR results. In addition,

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Akonni is now collaborating with a major company that is successfully selling high multiplex PCR kits for a number of tests.

Design and assemble a system that integrates sample preparation and microarray subciruits

Over the course of this one year project, we succesfully designed and assembled a sample-to-answer system, which included: a microfluidic cartridge with injection-molded pin valves, control of the cartridge pin valves, a fluidic subassembly, a unique rapid fluidic-based thermal cycler, a low-cost microarray optical subsystem based on a LED and a non-cooled CCD camera, isothermal hot air flow for APEX heating, and automated software that controlled all components of the system. This milestone was completed as proposed.

Upgrade the integrated cartridge with lyophilized reagents: Positive results were obtained using the Lyospheres, with the APEX Lyospheres displaying better performance than the PCR Lyospheres. However, studies in which Taq polymerases and PCR buffers were lyophilized independently revealed that the enzymes remained stable but the buffers became problematic. As a result of the extensive time in both the Lyosphere and integrated system development, implementing the Lyospheres for sample-to-answer system testing was not achieved. Nevertheless, automated rehydration of a Lyosphere in the cartridge was demonstrated. Future work will involve improving PCR buffer stabilization during lyophilization, or as an option, keep the buffer as wet chemistry since use of lyophilization is primarily to maintain enzyme stability.

Sample-to-answer testing on the integrated system

Whole blood was purified on the cartridge using an automated protocol. Additionally, PCR amplification was demonstrated on 1000 genomic copies on the automated cartridge system as well as isothermal APEX. Improved optical sensitivity is necessary to discriminate low copy numbers on cartridge. This requires optimization of materials, optical components such as the CCD camera, and/or the amplification (PCR and/or APEX) efficiency.

The sample-to-answer testing included the following sequence of steps that were successfully integrated and automated using the microfluidic cartridge after addition of a blood sample: introduction of bind buffer into sample tower, mixing sample with bind buffer using air, transport of sample mix to TruTip tower, toggle between the towers, dispense to waste, introduction of wash buffer to the TruTip tower, toggle between the towers, dispense to waste, introduction of elution buffer, and dispense to elution tower, add PCR mix to elution tower, dispense to PCR chamber on flow cell, thermal cycle with the Akonni bladder thermal cycler, flush PCR chamber with APEX buffer and add to APEX reservoir, thoroughly mix, add APEX reagent to array chamber, incubate at 65°C, wash, and image. We were able to demonstrate this using one SNP marker and not all six SNPs since the assay development required more time than planned, and further assay and hardware improvements are required to increase signal intensities on the array.

Due to the extensive troubleshooting for both assay and hardware development the integrated system was limited to demonstrating sample-to-answer results for one SNP eye color marker. This was achieved at the end of the project. Additional time and resources would have been required to obtain multiplex data and test results using mock forensic samples (de-identified blood with the known eye

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color phenotypes was purchased for this task). However, we were very encouraged to achieve our first automated, integrated data since this established a baseline for additional system improvements.

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, alpha unit 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 beta 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:

Phase III will continue defining protocols and assay chemistries, add additional SNP markers, and further refine the packaging of components and reagents in the integrated system. Plans are in progress to reconfigure the platform in Phase III to allow processing and analyzing up to 8 samples in parallel. Low cost molding of the disposables will be more cost-effective in the updated design. Developing an intuitive software and graphical user interface, and reducing the instrument footprint (approximately 2-3 cubic ft) remain high priorities. Protocols and assays will further be streamlined for minimum complexity, time, and cost. Most importantly, targeting a sample LOD of 0.5-1.0 ng is a priority for product development, and will be more realistic to achieve as system improvements are addressed. In addition, we look to complete transition assay reagents (*e.g.*, PCR, APEX) into the lyophilized format for long term storage and field deployment.

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The successful commercialization of an end-to-end SNP-typing platform has important implications for translating forensically important physical appearance DNA markers into a low-cost, user-friendly format. The most noteworthy is the unreliability of eyewitnesses. Our platform could confirm or reject an eyewitness description. The major benefit would be preventing misdirection of law enforcement resources by inaccurate eyewitness descriptions of a missing suspect or victim.

Given the fluidic process described herein, the same basic microfluidic format and manufacturing infrastructure could be applied to 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; 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. 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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VI. DISSEMINATION OF RESEARCH FINDINGS

Poster and Instrument Demonstration at the NIJ Conference, Arlington, VA, June 14-16, 2010,

Presentation to the DNA TWG. Washington, DC, May 5, 2010.

Cooney CG, Sipes D, Thakore N, Holmberg R, and Belgrader P. A valveless PCR microarray flow cell that supports on-chip washing. Lab-on-a-Chip (In Review)

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