Document Title: A Low Cost Microfluidic Microarray System for Typing SNPs

Author(s): Jennifer Reynolds, Ph.D., Christopher Cooney, Ph.D., Alex Kukhtin, Ph.D., Steve Garber, Nitu Thakore, Peter Qu, Jennifer Parker, Arial Bueno, Maria Belgrader, Phillip Belgrader Ph.D.

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Section I:

- Recipient name, phone, email and address:

Charles E. Daitch, Ph.D.
Tel: 301-524-7867
Email: cdaitch@akonni.com
Akonni Biosystems, Inc.
400 Sagner Avenue
Suite 300
Frederick, MD 21701

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- PI contact info (phone and email): Tel: 240-626-7191; jreynolds@akonni.com

Report Authors: Jennifer Reynolds, Ph.D., Christopher Cooney, Ph.D., Alex Kukhtin, Ph.D., Steve Garber, Nitu Thakore, Peter Qu, Jennifer Parker, Arial Bueno, Maria Belgrader, Phillip Belgrader Ph.D.

Abstract:

The goal of this work was to assemble and test an integrated microfluidic controlled microarray system for SNP-based typing of phenotypic markers. We previously demonstrated feasibility of Akonni's sample preparation, PCR, and gel drop microarray components as a complete solution for forensic SNP-typing applications. Components were packaged into prototype flow-through, microfluidic modules to demonstrate SNP discrimination on forensic samples. We then optimized protocols and assay chemistries for phenotype determination, and packaged components and reagents into an early prototype integrated system for automated, sample-to-answer results. This system consisted of the instrument (i.e., fluidic handling, Akonni Bladder Thermocycler, and Akonni imager) and disposable components (i.e., Akonni TruTip, Akonni PCR and TruArray flow cell chambers). This project advanced a breadboard prototype into an alpha unit, and expanded the assay panel to include phenotype markers for gender and ancestry.

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EXECUTIVE SUMMARY

Akonni Biosystems, Inc. was awarded a grant from the National Institute of Justice, Office of Justice Programs, in July 2011 to integrate Akonni's patented sample preparation (TruTip), thermocyling, and gel drop microarray (TruArray™) technologies for the typing of SNPs for forensic applications. Eye color SNPs initially served as the model system with the panel expanding to include markers for gender and ancestry. These additional markers include both SNPs and DIPs (deletion/insertion polymorphisms). Over the last few years, there has been a tremendous understanding of the linkage of human physical appearance to SNP and DIP markers. While there can be many of these markers associated with a specific aspect of physical appearance, such as eye color, the markers 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.

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
- walk-away push-button operation (including the sample preparation)
- low-cost
- disposable cartridges for closed PCR and SNP typing (substantially reduced chances of cross-contamination)
- small-footprint
- portable for field or mobile lab use
- flexible platform (amenable to a wide range of commercial applications)

Akonni Biosystems has an exclusive license to a gel drop microarray 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 drop microarrays for protein, nucleic acid and on-chip PCR analyses. The fundamental difference between Akonni's 3-dimensional gel drop arrays and other microarrays is that individual polymeric gel drops literally create an array of 3-dimensional "test tubes." Gel drops are attached to a solid support (glass or plastic), but the microarray capture probes are covalently crosslinked to the polymer backbone instead of the two-dimensional substrate surface.

Since forensic typing laboratories of varying resources and capacity must make use of common forensic techniques, simplicity and reproducibility are key factors to consider. Technologies that are easily implemented, easy to standardize, and easy to quality control are therefore in demand. The cost of a technique, both in labor and reagents, also significantly affects its use and thus effectiveness. The development of a SNP-typing technology that combines sensitivity, discrimination power, analytical accuracy and robustness with operational simplicity, readiness for standardization, rapid turn-around time, multiplexing capacity, and low unit costs will be of substantial impact to this community.

We proposed to advance an integrated sample-to-answer system for typing eye color SNP markers while expanding the phenotype markers for other physical traits. The specific technical objectives and milestones for the project were:

1. Design and assemble integrated alpha units;
2. Manufacture molded cartridges;
3. Expand SNP panel and lyophilized assays; and
4. Sample-to-answer testing on the integrated system.

Consistent with our roadmap, the project culminated in an alpha system that utilized a microfluidic cartridge inserted into an instrument to perform automated sample-to-answer typing of eye color SNPs and ancestry DIPs.

Implications for Policy and Practice

The successful commercialization of a sample-to-answer SNP/DIP-typing platform has important implications for translating forensically important physical appearance DNA markers into a low-cost, user-friendly format to serve as an objective alternative to unreliable witnesses. 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 testing) are today becoming a new and emerging application area that could benefit greatly from an integrated sample-to-answer rapid test platform.

MAIN REPORT

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 as 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 facial features, hair type and growth pattern, height, etc. Currently, the widely used STR kits include the amelogenin deletion (non-STR marker) to determine an individual's gender. 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 cost will provide a new tool to aid in apprehending a perpetrator or identifying a victim, thereby 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.

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

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

Background to Akonni Technologies

Gel Drop Microarray. Akonni Biosystems has an exclusive license to a mature gel drop 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 drop microarrays for protein, nucleic acid and on-chip PCR analyses (e.g., [4-14]). The fundamental difference between Akonni's 3-dimensional gel drop arrays and other commercially available substrates is that individual polymeric gel drops 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 drop 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.

Hybridization for SNP Typing: SNP and DIP (deletion/insertion polymorphsism) typing of Y-chromosome markers (M2, defining most African Americans, M170, defining most Caucasians, and M175, defining most Asians) and amelogenin markers was demonstrated. Multiplex PCR and microarray hybridization was performed on five de-identified samples. Typing was based on probe signal ratios for each Y-chromosome haplotype marker and presence or absence of positive probe signal for each amelogenin marker. Table 2 provides a summary of the results.

Table 2. Summary of Microarray Hybridization Results for Y-chromosome Haplotype and Amelogenin Markers

	M2_A/ M2_G	M170_A/ M170_C	M175_D/ M175I	AmelX	AmelY	Identified Alleles
Sample A	2.03	1.21	0.46	+	+	M2_A, M170_A, M175_I, AmelX, AmelY
Sample C	0.41	1.35	0.50	+	+	M2_G, M170_A, M175_I, AmelX,AmelY
Sample E	1.80	0.48	0.50	+	+	M2_A, M170_C, M175_I, AmelX,AmelY
Sample CEZ	1.49	1.17	0.35	+	+	M2_A, M170_A, M175_I, AmelX,AmelY
Sample F	N/A	N/A	N/A	+	-	Chr4_A, AmelX

Chr4_A stands for allele A of the SNP rs279844. Y-chromosome: values represent the ratio of probe signals for each allele; amelogenin: probe signal intensities scored as positive (+) or negative (-) for each allele. Determined genotypes for each sample are indicated in the last column.

We applied the TruArray test to discriminate eye color SNPs (using the six SNP loci listed in Table 1) on samples provided by the Massachusetts State Police Forensic Services Group. As summarized in Table 3, the genotyping and predictive phenotyping results showed good correlation with the actual phenotypes. Each genotype in Table 3 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.

Table 3. Predicting Eye Color Phenotypes

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

TruTip Sample Preparation Module: For sample preparation, Akonni uses a modified, patented pipet tip, called TruTip, containing a rigid, porous silica extraction matrix to bind and elute nucleic acid.

Comparisons of the quantitative real-time PCR results on DNA purified from blood using Akonni's TruTip (operated by Rainin Electronic Pipettor) and a standard Qiagen kit (requiring centrifugation) indicated that both methods exhibited the same recoveries of DNA. In addition, TruTip was faster, utilizing a 4-minute bind, wash, dry, and elution process compared to 20 minutes for Qiagen. The TruTip and Qiagen kits were also evaluated on saliva samples. DNA recoveries of processed triplicate saliva samples resulted in 4.6 µg for Qiagen and 6.0 µg for the TruTip.

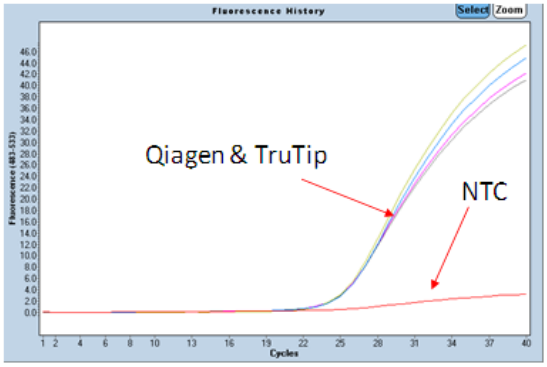

Figure 1. The TruTip sample preparation module provides the same sample preparation performance as the commercial Qiagen kit on whole blood. Shown are real-time PCR results of 100 µl of processed duplicate samples for each preparation method.

Flow Cell PCR/Microarray Module: For PCR and microarray reaction chambers, Akonni employs flow cells consisting of proprietary materials and flow dynamic properties. Flow cells allow the filling and emptying of chambers without bubble formation. Substantial effort went into identifying, testing, and optimizing PCR-friendly materials that displayed proper fluidic flow and low fluorescence background for imaging.

To address the non-uniformity in temperature distributions and slow ramp times associated with commercial slide-based thermocyclers, we explored an alternative fluidic-based approach. This approach consisted of two recirculating fluidic pathways that alternated between a bypass loop and a loop that has a pair of inflatable and compliant "bladders." One pathway was held constant at the high (denaturing) temperature and the other pathway was held constant at the low (annealing and extension) temperature. Before startup when the working fluids were not recirculating, the bladders were relaxed, allowing the flow cell to be easily inserted between the bladder pair. During initialization, the recirculating fluids began to flow and pressurize the "bladder pair", which consequently expanded and made tight contact with the flat reaction chamber of the flow cell. During this initialization state, two separate in-line heating units reached the appropriate temperatures (*e.g.*, 95°C and 60°C). Denaturation, which followed the initialization state, was achieved by switching three-way valves, so that the high temperature fluid flowed through the "bladder pair" loop, and the low temperature working fluid flowed through the bypass loop. During thermocycling, which followed denaturation, the valves were 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 flowed through the "bladder

pair" loop and the high temperature working fluid flowed through the bypass loop), and these states continued to switch throughout the thermocycling protocol.

Results: Early work involved automating TruTip sample preparation. This was accomplished under computer control in a cartridge format on a sample of 50 µL of blood. Real-time PCR results on the extracted, purified DNA sample displayed a strong positive PCR signal (Figure 2). More significantly, this purified DNA was correctly genotyped for eye color using the TruArray™ test (Figure 3).

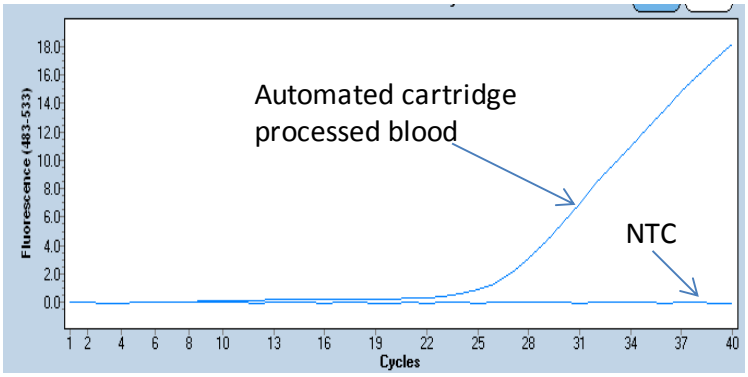

Figure 2. Real-time PCR analysis of DNA purified by automated TruTip.

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 3. Automated cartridge-purified DNA was used for manual PCR (20 ng DNA) and 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.

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 the project was to design and assemble an alpha level prototype to demonstrate automated sample-to-answer typing of phenotype markers. The alpha unit design was

based on cost-benefit, trade-off and marketing analyses to follow the production path with a system compatible with a broad range of market applications to leverage the cost benefit of scaled-up manufacturing.

II. METHODS

Genomic DNA samples: Genomic DNA was procured from NIST and Dr. Mark Shriver at Penn State University. Blood was purchased from Valley Biomedical.

III. RESULTS

The integrated system architecture was designed to be flexible to allow iterative changes, upgrades, and reconfiguration as we proceeded toward an alpha-level system that can process multiple samples. Akonni intellectual property (TruTip, flow cell, bladder thermocycler, gel-drop microarray and LED-based reader) were at the core of the system.

An assay was developed that utilized multiplex amplification on gel-spot microarrays to discriminate these SNPs. Three genomic DNA samples were used: DNA A from NIST SRM 2372 Human DNA quantitation kit, DNA extracted from blood using the manual TruTip protocol, and DNA extracted from blood using the TruTip in the automated fluidic subsystem. DNA in the amounts of 2 and 10 ng was tested for each sample with three replicate experiments performed in each test.

Figure 4 contains agarose gel results of PCR products.

		V (µl)
	Upper Part (Low-temp 2-step Multiplex PCR with 2 ng DNA)	
1	Marker	10
2	DNA A (NIST SRM 2372 kit) 2 ng (#1)	10
3	DNA A (NIST SRM 2372 kit) 2 ng (#2)	10
4	DNA A (NIST SRM 2372 kit) 2 ng (#3)	10
5		
6	DNA from Blood (TruTip manual extraction) 2 ng (#1)	10
7	DNA from Blood (TruTip manual extraction) 2 ng (#2)	10
8	DNA from Blood (TruTip manual extraction) 2 ng (#3)	10
9		
10	DNA from Blood (380 automated extraction) 2 ng (#1)	10
11	DNA from Blood (380 automated extraction) 2 ng (#2)	10
12	DNA from Blood (380 automated extraction) 2 ng (#3)	10
	LowerPart (Low-temp 2-step Multiplex PCR with 10 ng DNA)	
13	Marker	10
14	DNA A (NIST SRM 2372 kit) 10 ng (#1)	10
15	DNA A (NIST SRM 2372 kit) 10 ng (#2)	10
16	DNA A (NIST SRM 2372 kit) 10 ng (#3)	10
17		
18	DNA from Blood (TruTip manual extraction) 10 ng (#1)	10
19	DNA from Blood (TruTip manual extraction) 10 ng (#2)	10
20	DNA from Blood (TruTip manual extraction) 10 ng (#3)	10
21		
22	DNA from Blood (380 automated extraction) 10 ng (#1)	10
23	DNA from Blood (380 automated extraction) 10 ng (#2)	10
24	DNA from Blood (380 automated extraction) 10 ng (#3)	10

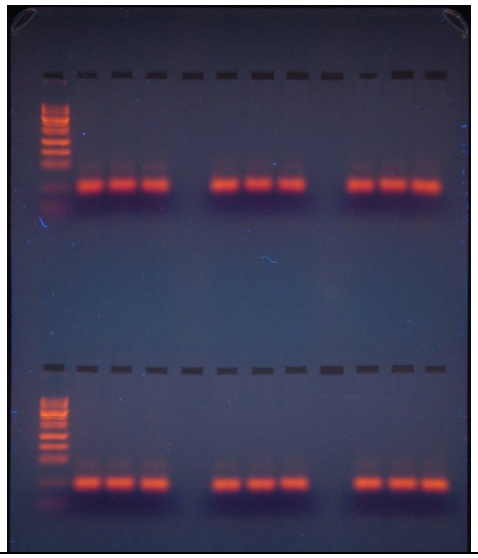

Figure 4. Multiplex amplifications.

Figure 5 shows TruArray assay signals generated from the respective multiplex PCR conditions. These signals are averages calculated from 3 replicate experiments for each sample with error bars indicating standard deviation. TruArray™ signals remain consistent for 2 and 10 ng of DNA. The hybridization signals are obtained with an acquisition time of 0.2 s. Figure 6 displays genotype ratios for the TruArray assays.

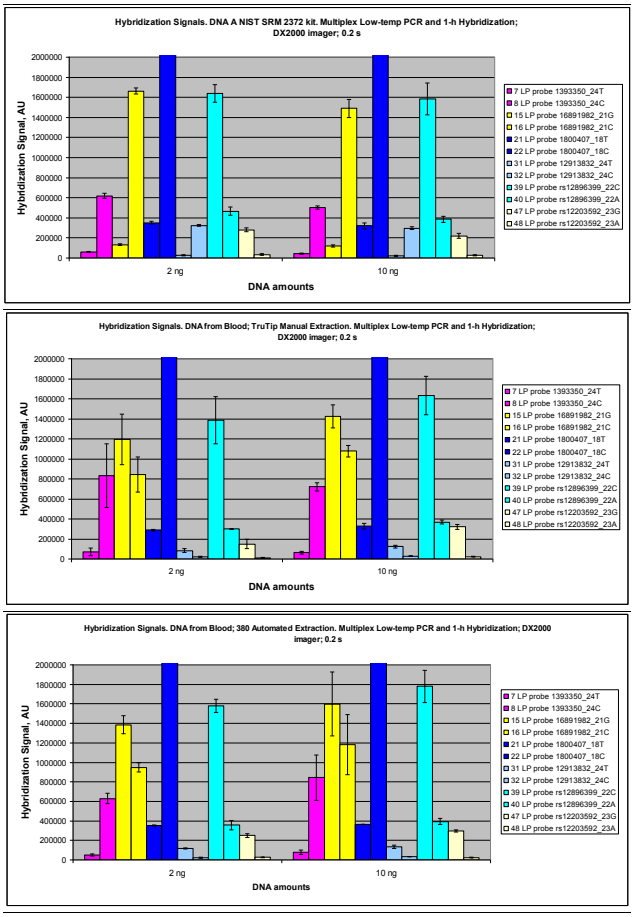

Figure 5. Allele signals generated using TruArray assays.

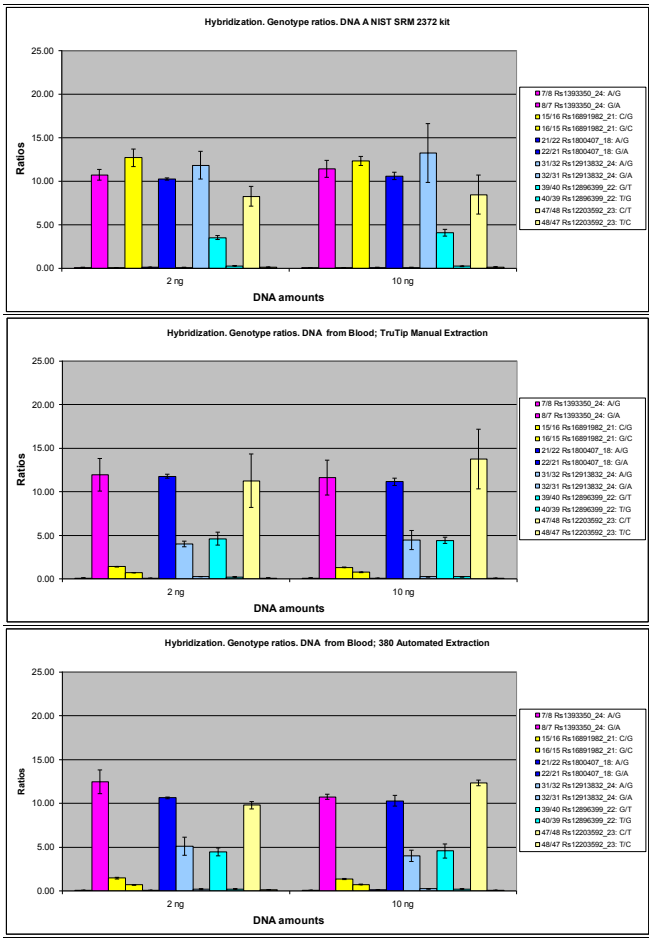

Figure 6. Genotype ratios for TruArray assays**.**

Table 4 summarizes the data for eye color SNP typing for the TruArray assays. A genotype call was considered confirmed when all three replicate experiments produced consistent data for the probe pair of interest. A single allele was considered to be present when a ratio between probe signals in the corresponding probe pair was >2. If the ratio was <2, the presence of both alleles was called.

In summary, the multiplex amplification followed by direct microarray hybridization provides reliable and robust genotyping results. These findings along with single-chamber PCR/hybridization flow cell advances factored into the hardware design review for the alpha unit.

Table 4. Summary of Genotyping Produced by TruArrays

Hybridization assay		DNA A (NIST SRM 2372 kit)		DNA Blood TruTip manual extraction		DNA Blood 380 automated extraction	
Probe/Pro	Description	2 ng	10 ng	2 ng	10 ng	2 ng	10 ng
7/8	Rs1393350_24: A/G	G/G	G/G	G/G	G/G	G/G	G/G
8/7	Rs1393350_24: G/A						
15/16	Rs16891982_21: C/G	G/G	G/G	C/G	C/G	C/G	C/G
16/15	Rs16891982_21: G/C						
21/22	Rs1800407_18: A/G	G/G	G/G	G/G	G/G	G/G	G/G
22/21	Rs1800407_18: G/A						
31/32	Rs12913832_24: A/G	G/G	G/G	A/A	A/A	A/A	A/A
32/31	Rs12913832_24: G/A						
39/40	Rs12896399_22: G/T	G/G	G/G	G/G	G/G	G/G	G/G
40/39	Rs12896399_22: T/G						
47/48	Rs12203592_23: C/T	C/C	C/C	C/C	C/C	C/C	C/C
48/47	Rs12203592_23: T/C						

To enhance the sensitivity of the existing eye-color SNP assay, some probes were redesigned to minimize cross-hybridization with forward Cy3-labeled primers used in the assay. In addition, probes for the detection of amelogenin X and Y markers were added to allow gender determination. Therefore, with the introduction of amelogenin primers, the existing 6-plex PCR was converted to 7-plex PCR for an eye-color/gender SNP test.

Table 5. Mixes of Synthetic Oligonucleotides Simulating Different Sample Genotypes

SNP	Mix I	Mix II	Mix III
Rs1393350	GG	AA	GA
Rs16891982	CC	GG	CG
Rs1800407	GG	AA	GA
Rs12913832	AA	GC	AG
Rs12896399	GG	TT	GT
Rs12203592	CC	TT	CT

Long synthetic oligonucleotide mixes that simulated different sample genotype templates were subjected to multiplex PCR to identify the best performing microarray probes, determine the most optimal hybridization conditions, and establish threshold ratios for calling the eye color SNP alleles. Three sets of oligonucleotide mixes (Table 5) representing the three possible genotypes (two homozygous, one heterozygous) for each eye color SNP were PCR amplified and hybridized to the array at different temperatures.

The 7-plex eye color/gender SNP assay was evaluated using flow cells (PCR and hybridization in the same chamber) and the control method (conventional tube amplification followed by hybridization). 10 ng of DNA from two different samples was tested. As shown in Figure 7 and Table 6, the probe signals and ratios determined using the flow cell and the control method were similar for each sample.

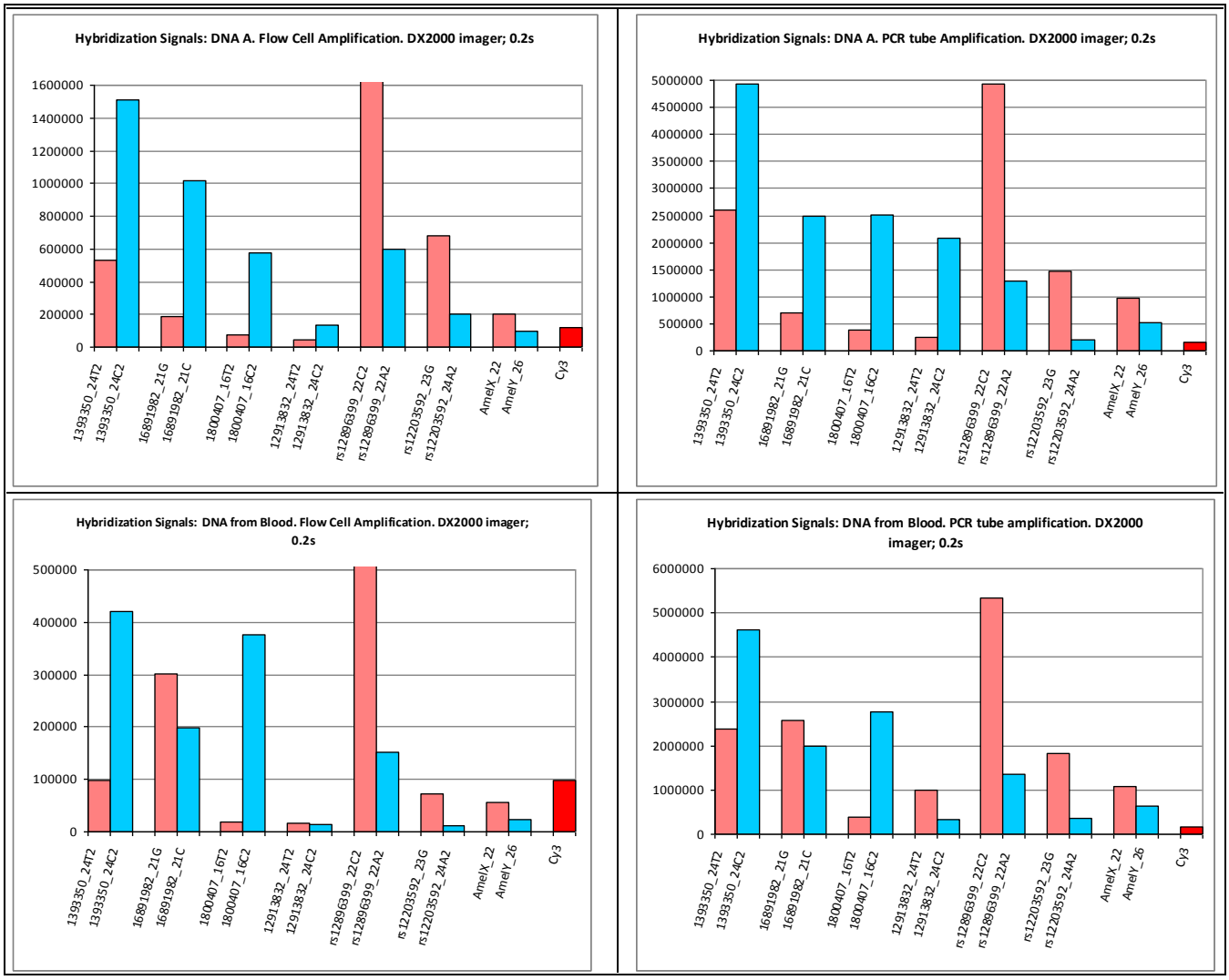

Figure 7. Hybridization signals for the eye color/gender SNP assay. Signals generated from (left panels) the flow cell (same chamber PCR/hybridization) or (right panels) conventional PCR tube amplification with separate hybridization. Sample tested were (upper panels) DNA A from NIST SRM 2372 kit or (lower panels) DNA extracted from blood using the automated subsystem.

Table 6. Summary of Genotyping Ratios Generated from Flow Cells and Tube Control

DNA A. Flow cell amplification	Ratios			
	A1/A2	A2/A1	Threshold	Genotype
rs1393350; A1=A; A2=G	0.35	2.86	1.75	GG
rs16891982; A1=C; A2=G	0.19	5.40	2	GG
rs1800407; A1=A; A2=G	0.13	7.80	2	GG
rs12913832; A1=A; A2=G	0.24	4.24	1.75	GG
rs12896399; A1=G; A2=T	4.10	0.24	3	GG
rs12203592; A1=C; A2=T	3.42	0.29	1.75	CC

	Amelogenin (/Cy3)		
			Threshold
Amel_X	1.70	PRESENT	0.2
Amel_Y	0.82	PRESENT	0.2

DNA A. PCR tube amplification	Ratios			
	A1/A2	A2/A1	Threshold	Genotype
rs1393350; A1=A; A2=G	0.53	1.90	1.75	GG
rs16891982; A1=C; A2=G	0.28	3.58	2	GG
rs1800407; A1=A; A2=G	0.16	6.38	2	GG
rs12913832; A1=A; A2=G	0.45	2.22	1.75	GG
rs12896399; A1=G; A2=T	3.82	0.26	3	GG
rs12203592; A1=C; A2=T	7.01	0.14	1.75	CC

	Amelogenin (/Cy3)		
			Threshold
Amel_X	6.03	PRESENT	0.2
Amel_Y	3.17	PRESENT	0.2

DNA from Blood. Flow cell amplif	Ratios			
	A1/A2	A2/A1	Threshold	Genotype
rs1393350; A1=A; A2=G	0.23	4.30	1.75	GG
rs16891982; A1=C; A2=G	1.52	0.66	2	CG
rs1800407; A1=A; A2=G	0.05	19.35	2	GG
rs12913832; A1=A; A2=G	2.14	0.47	1.75	AA
rs12896399; A1=G; A2=T	6.50	0.15	3	GG
rs12203592; A1=C; A2=T	6.11	0.16	1.75	CC

	Amelogenin (/Cy3)		
			Threshold
Amel_X	0.57	PRESENT	0.2
Amel_Y	0.24	PRESENT	0.2

DNA from Blood. PCR tube amplif	Ratios			
	A1/A2	A2/A1	Threshold	Genotype
rs1393350; A1=A; A2=G	0.51	1.96	1.75	GG
rs16891982; A1=C; A2=G	1.29	0.77	2	CG
rs1800407; A1=A; A2=G	0.14	6.97	2	GG
rs12913832; A1=A; A2=G	2.68	0.37	1.75	AA
rs12896399; A1=G; A2=T	3.97	0.25	3	GG
rs12203592; A1=C; A2=T	4.93	0.20	1.75	CC

	Amelogenin (/Cy3)		
			Threshold
Amel_X	7.07	PRESENT	0.2
Amel_Y	4.07	PRESENT	0.2

NIST DNA A (upper panels) and DNA from blood (lower panels), flow cell (left panels), tube control (right panels)

Additional performance of the 7-plex eye color/gender SNP assay was tested on samples. 10 ng of each sample was subjected to the control PCR tube amplification method to focus result interpretation strictly on assay chemistry and not new hardware. Genotype calls for the samples are shown in Table 7. According to Dr. Shriver, the X and Y amelogenin typing matched the gender data on record for the samples. While eye color information for these samples was not available, this does show the correspondence of the Flow Cell results to the tube controls.

Table 7. Summary of Genotype Results on DNA Samples from Different Individuals

	50730	60313	50939	50830	50741	60224	50881	60003	64040	50759	64519	60329	60325	60032	64455	64259	64038	64598	64050	64094
rs1393350; A1=A; A2=G	GG	GG	GG	GG	GG	GG	AG	GG	GG	GG	GG	AG	AG	GG	GG	GG	GG	GG	AG	AG
rs16891982; A1=C; A2=G	CC	GG	CC	CC	CC	CC	CC	CC	CC	CC	CC	CG	CC	GG	CC	GG	CG	CC	CG	CG
rs1800407; A1=A; A2=G	GG	GG	GG	GG	GG	GG	GG	AG	GG	GG	AG	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs12913832; A1=A; A2=G	AA	AA	AA	AA	AA	AA	AA	GG	AA	AA	AG	AA	AG	AA	AA	AG	AG	AA	AA	AA
rs12896399; A1=G; A2=T	GG	GT	GG	GG	GG	GT	GG	GG	GG	GG	GG	GG	GG	GG	GG	TT	GG	GG	GG	GG
rs12203592; A1=C; A2=T	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CT	CC	CC	CC
Amel_X	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
Amel_Y	PRESENT	ABSENT	PRESENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	PRESENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT

Expanded Panel

The 7-plex eye color/gender panel that includes the six eye color SNPs and the amelogenin DIP for gender identification is summarized in Table 8.

Table 8. Eye Color/Gender Markers

Eye Color SNPs			
SNP id	**Chr**	**Chromosome Position**	**alleles**
rs12913832	15	26039213	G,A
rs1800407	15	25903913	C,T
rs12896399	14	91843416	T,G
rs16891982	5	33987450	G,C
rs1393350	11	88650694	G,A
rs12203592	6	341321	C,T
rs3082850	1	53145226	C,T
Amelogenin DIP			
	Chrom	**Chromosome Position**	**Alleles**
	x	11314994	(-)
	y	6737888	AAAGTG

To further expand the assay to include ancestry phenotype information, the Shriver Laboratory at Penn State University performed extensive bioinformatics to select additional phenotype markers. While additional SNP markers to deduce phenotype exist, there were also many DIP (deletion/insertion polymorphisms). Since DIPs exhibit >1 nucleotide difference between alleles, they are potentially more robust than SNPs for hybridization-based discrimination. Therefore, Table 9 includes 8 DIP AIMs (ancestry informative markers), and lists the frequency of each of the DIP AIM alleles in European, African, and East Asian populations.

The likelihood ratios (Table 10) illustrate the statistical significance for correctly classifying a person as belonging to the population from which he was sampled. These values are computed across loci to compute the multi locus likelihood ratios. For example a person showing the most common European multi locus genotype has the multi locus genotype rs16725*AA, rs1611004*BB, rs2307527*AB, rs2307592*BB, rs3027972*AA, and rs3034080*AA which is found at a frequency of 0.081 in European Americans. Since this genotype is expected to be found at a much lower frequency in African Americans (0.000000087), there is substantial support for the source population being European American when this genotype is found. The statistical confidence in this conclusion is expressed as the log likelihood ratio and equals 5.97 in this case.

Probes and primers have been designed for the DIP AIMs, and DIP microarrays were then printed. Table 11 shows the assay performance of these DIP markers across 12 samples, received from Dr. Shriver, at 10 ng. AA represents homozygous for the mutant, AB is heterozygous, and BB is homozygous for wildtype. The TruArray data shows a concordance of 99% with fragment analysis using capillary electrophoresis (analyzed by Operon).

Table 9. DIP AIMs

snp_id	alleles	loc_pop_id	freq	loc_pop_id	freq	delta values
rs16725	TTTGT,-	African	0.94	Japanese	0.45	0.49
rs1611004	GACT,-	European	0.1	African	0.7	0.6
rs2307527	TGTGA,-	European	0.42	African	0.92	0.5
rs2307592	GACT,-	European	0.17	African	0.72	0.55
rs2307592	GACT,-	African	0.72	Japanese	0.11	0.61
rs3027972	TGT,-	European	0.78	African	0.024	0.756
rs3027972	TGT,-	African	0.024	Japanese	0.731	0.707
rs3034080	TGG,-	European	0.961	African	0.406	0.555
rs3034080	TGG,-	African	0.406	Japanese	0.966	0.56
rs3063668	AA,-	African	0.865	Japanese	0.252	0.613
rs3063668	AA,-	European	0.756	Japanese	0.252	0.504
rs66752578	AG,-	CAUC1	0.822581	AFR1	0	0.822581

Table 10. Likelihood ratios of ancestry markers.

	European/African			African/European			European/Japanese		
SNP ID	AA	AB	BB	AA	AB	BB	AA	AB	BB
rs16725	0.60	3.49	20.25	1.66	0.29	0.05	2.63	0.80	0.24
rs1611004	0.02	0.43	9.00	49.00	2.33	0.11	0.05	0.36	2.68
rs2307527	0.21	3.31	52.56	4.80	0.30	0.02	0.40	1.09	2.91
rs2307592	0.06	0.70	8.79	17.94	1.43	0.11	2.39	1.44	0.87
rs3027972	1056.25	7.33	0.05	0.00	0.14	19.68	1.14	0.87	0.67
rs3034080	5.60	0.16	0.00	0.18	6.43	231.98	0.99	1.14	1.32

Table 11. TruArray DIP Analysis

		TruArray Analysis											
Sample ID		50730	50939	50830	50741	60003	50759	60325	60032	64038	64598	64050	64094
Ancestry		African American	African American	African American	African American	African American	African American	African American	African American	Brazilian	Brazilian	Brazilian	Brazilian
rs16725	101/102; 102/101	BB	BB	BB	BB	BB	BB	BB	BB	AB	AB	BB	BB
rs1611004	121/122; 122/121	BB	AB	AA	AB	BB	AB	AA	BB	AA	AB	AA	AB
rs2307527	138/139; 139/138	BB	AA	BB	AB	BB	AB	AB	BB	AB	AA	AA	BB
rs2307592	141/142; 142/141	AB	AB	BB	BB	AB	AB	BB	AB	AB	AA	AB	AA
rs3027972	151/152; 152/151	AA	AA	AB	AB	AA	AA	AB	AB	BB	BB	AA	AB
rs3034080	158/159; 159/158	BB	AB	AA	BB	BB	BB	AB	BB	AB	AB	BB	BB
rs3063668	171/172; 172/171	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
rs66752578	193/194; 194/193	BB	BB	BB	BB	BB	BB	BB	AB	BB	AB	BB	AB
Correct calls		8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8	7 out of 8	8 out of 8	8 out of 8	8 out of 8	8 out of 8
		Fragment analysis data											
Sample ID		50730	50939	50830	50741	60003	50759	60325	60032	64038	64598	64050	64094
rs16725	101/102; 102/101	BB	BB	BB	BB	BB	BB	BB	BB	AB	AB	BB	BB
rs1611004	121/122; 122/121	BB	AB	AA	AB	BB	AB	AA	BB	AA	AB	AA	AB
rs2307527	138/139; 139/138	BB	AA	BB	AB	BB	AB	AB	BB	AB	AA	AA	BB
rs2307592	141/142; 142/141	AB	AB	BB	BB	AB	AB	BB	AB	AB	AA	AB	AA
rs3027972	151/152; 152/151	AA	AA	AB	AB	AA	AA	AB	AB	BB	BB	AA	AB
rs3034080	158/159; 159/158	BB	BB	AA	BB	BB	BB	AB	BB	AB	AB	BB	BB
rs3063668	171/172; 172/171	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
rs66752578	193/194; 194/193	BB	BB	BB	BB	BB	BB	BB	BB	BB	AB	BB	AB

Thus, an expanded panel of the SNP and DIP markers should allow predicting an individual's eye color, ancestry, and gender. This should provide valuable lead information to help apprehend a perpetrator, particularly when there are no or unreliable eyewitnesses.

Figure 8 shows the results of the standard wet reagents to the lyophilized reagent using 10 ng of a sample. The ratios are comparable for the two preparations, resulting in correct genotyping for the 7 plex SNP eye color and amelogenin DIP assay.

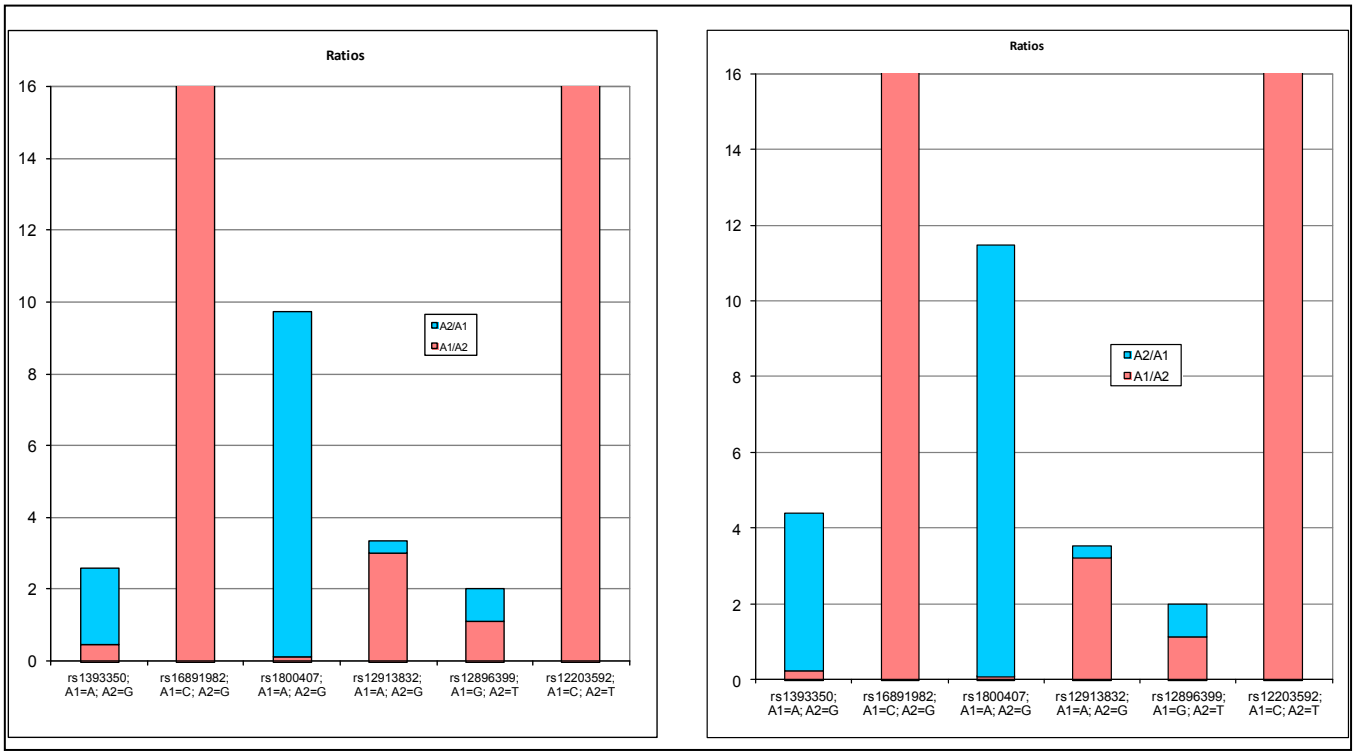

Figure 8. Six-plex SNP eye color assay using **(left)** Qiagen wet reagents **(right)** lyophilized reagent.

Table 12. Comparison of SNP Genotypes for Wet and Lyophilized Reagents

Eye Color SNPs		
SNP id	**Wet Reagent Genotype**	**Lyophilized Reagent Genotype**
rs12913832	AA	AA
rs1800407	GG	GG
rs12896399	GT	GT
rs16891982	CC	CC
rs1393350	GG	GG
rs12203592	GT	GT
Amelogenin DIP		
LP Probe AmelX_22	Present	Present
LP Probe A_26melY	Present	Present

Automated Sample-to-Answer Testing

The alpha unit includes an instrument and disposable. The instrument includes Fluidic, Thermocycler, and Optical subsystems. The first step in this phase involved an extensive design review to address components, modules and subsystems to meet marketing and manufacturing requirements. A significant effort has been devoted to assembling, debugging, and optimizing the alpha unit.

Testing using the automated system was performed on 50-µL samples of blood. Samples were loaded into the disposable, which was placed on the instrument. The sequence of automation steps was as follows:

1. DNA was extracted and purified from the blood using the on-board TruTip
2. Purified DNA was mixed with 6-plex eye color PCR reagent (wet chemistry)
3. PCR mix was moved to the PCR/microarray chamber in the flow cell cartridge
4. Coupled PCR/microarray hybridization was performed on the bladder thermocycler
5. The microarray was imaged for analysis

The results in Figure 9 and Table 13 were produced by running duplicates of four samples (two brown-eyed samples and two blue-eyed samples). The samples from the blue-eyed individuals exhibited GG genotypes, while the brown-eyed individuals displayed CG and CC genotypes.

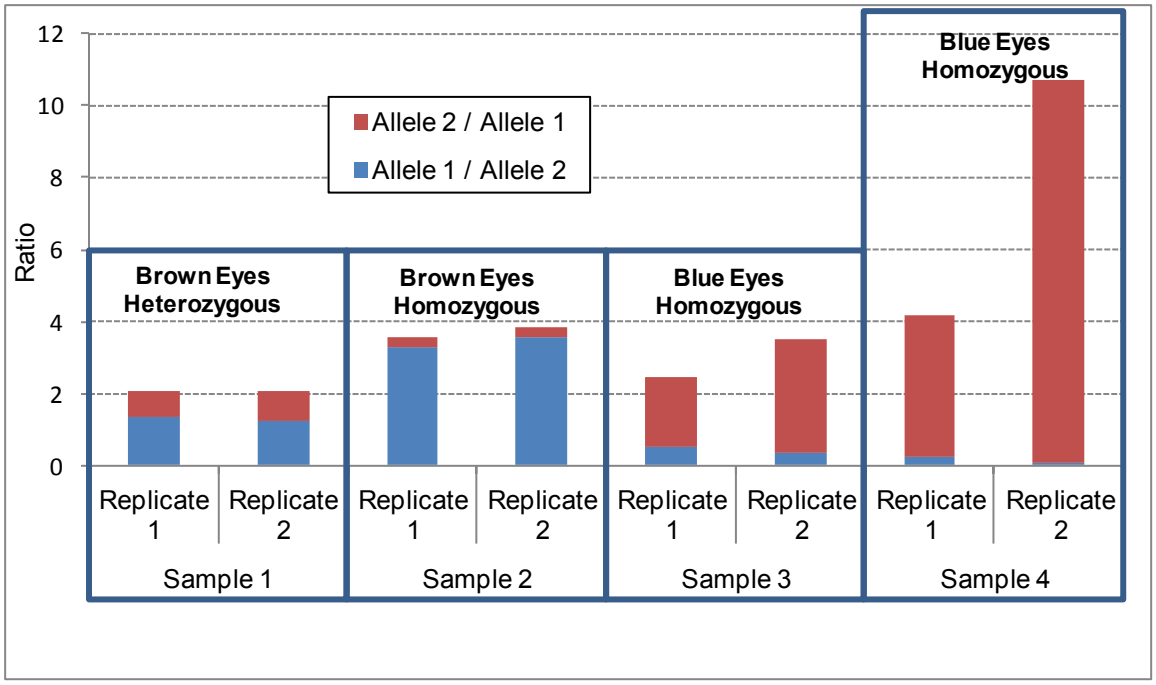

Figure 9. An example of an automated sample-to-answer eye color typing result for SNP rs16891982 obtained from duplicates of four samples (two brown eyed samples and two blue eyed samples) run on the automated hardware. The relative signal intensities for each allele were used to determine the genotype of each SNP in Table 12.

Table 13. Genotyping results from data in Figure 9 for 4 blood samples using a ratio threshold of 1.5.

Sample	Phenotype (Eye Color)	Replicate	Automated System	Manual Tube
1	Brown	1	CG	CG
1	Brown	2	CG	
2	Brown	1	CC	CC
2	Brown	2	CC	
3	Blue	1	GG	GG
3	Blue	2	GG	
4	Blue	1	GG	GG
4	Blue	2	GG	

We proceeded to test samples for the six eye color SNPs. Figure 10 and Table 14 depict an example of a successful typing result on blood for six eye color SNPs using the automated system.

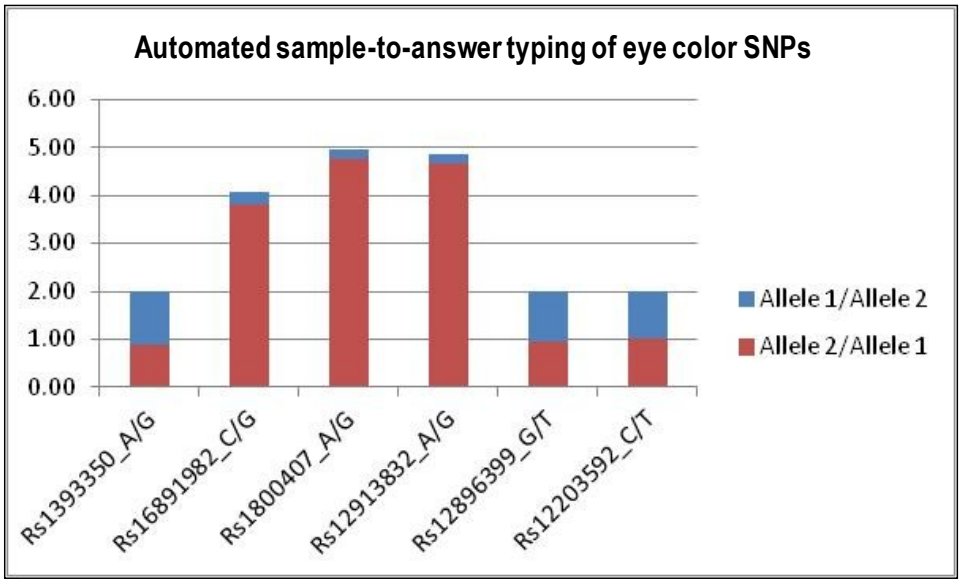

Figure 10. An example of an automated sample-to-answer eye color typing result obtained from 50-µl of whole blood. The relative signal intensities for each allele were used to determine the genotype of each SNP in Table 14.

Table 14. Genotyping Results on a Brown-eyed Individual.

Common Allele	Minor Allele	SNP ID	E381_Brown-Eye, Male	
			Automated System	Manual Tube Amplification
G	A	rs1393350	AG	AG
C	G	rs16891982	GG	GG
G	A	rs1800407	GG	GG
A	G	rs12913832	GG	GG
G	T	rs12896399	GT	GT
C	T	rs12203592	CT	CT

Samples were introduced into the automated system, and automatically processed and analyzed. Analysis include automated detection and analysis of spots. Figure 11 shows the ratios plotted for samples that are wild-type for the deletion allele. The strong discrimination is the reason the blue bar, representing the deletion allele, is low and difficult to see on many of the samples. Table 15 shows the comparison alongside the fragment analysis using capillary electrophoresis that were analyzed by Operon.

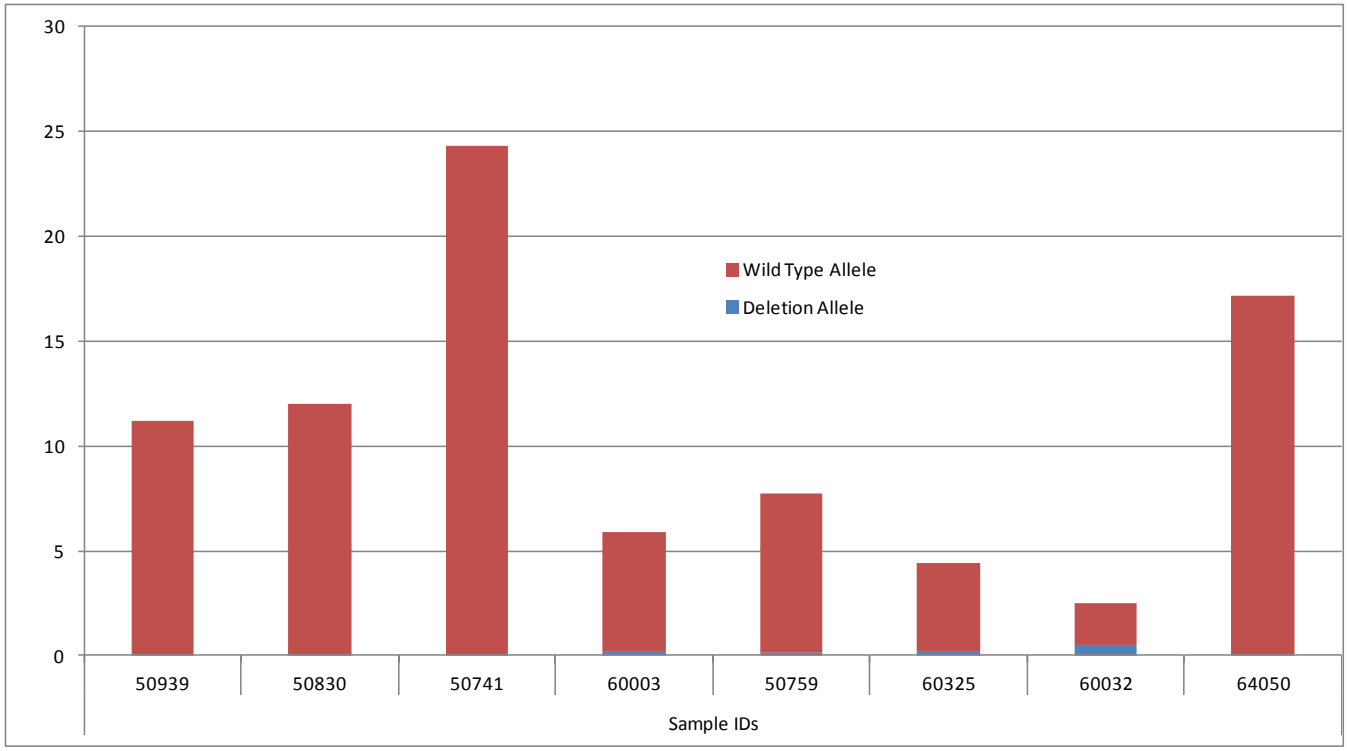

Figure 11. Automated processing across multiple samples for ancestry marker rs66752578.

Table 15. Genotyping results from data in Figure 10.

Sample	Automated System	Fragment Analysis
50539	BB	BB
50830	BB	BB
50741	BB	BB
60003	BB	BB
50759	BB	BB
60325	BB	BB
60032	BB	BB
64050	BB	BB

Sample-to-answer Demonstration to an External Collaborator

To externally validate the system, we invited Dr. Shriver to participate in on-site testing of the system using a provided sample. The system automatically processed the sample and an automated report displayed that the correct genotype for the rs2307527 African/European ancestry marker in his presence.

IV. CONCLUSION

Discussion of findings

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

Here, we summarize the results for each major task, and how well we met the objectives.

Design and assemble integrated alpha units

Over the course of this project, we succesfully designed, configured, and assembled a low-complexity sample-to-answer system, which included: a microfluidic cartridge with injection-molded parts, a fluidic subassembly, a rapid fluidic-based thermocycler, a low-cost microarray optical subsystem and software control of all components.

Expand SNP panel and scale up lyophilized assay production

The eye color SNP panel was expanded to include the amelogenin DIP as a 7-plex assay to predict eye color and gender. Ancestry phenotype information, developed at the Shriver Laboratory at Penn State University, were ported to Akonni's TruArray DIP chip assay. While additional SNP markers to deduce phenotype exist, there were also many candidate DIPs. Since DIPs exhibit >1 nucleotide difference between alleles, they are potentially more robust than SNPs for hybridization-based discrimination. Assays and microarrays for candidate DIP AIMs were designed, tested and verified to give the expected results when compared with the reference method of capillary electrophoresis.

Sample-to-answer testing on the integrated system

Whole blood was successfully processed and analyzed on an automated system. The sample-to-answer testing included the following sequence of steps that were automated after addition of a blood sample: mixing sample with bind buffer, extracting and binding DNA in sample to TruTip matrix, washing the TruTip matrix, drying the TruTip matrix, eluting the DNA from the TruTip matrix, mixing DNA with PCR mastermix, dispensing the PCR mixture into a flow cell PCR/microarray chamber in the cartridge , performing coupled PCR and hybridization using a bladder thermocycler, washing the chamber, drying the chamber, and imaging the microarray in the chamber. We were able to demonstrate this using all six

eye color SNPs. We additionally demonstrated correct identification of DIPs of four different samples in duplicate, on the sample-to-answer integrated system. Finally, as external validation, Dr. Shriver took part in an automated sample to answer DIP test.

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 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 production system that 1) is based on user specifications and requirements, 2) consists of components that are subjected to rigorous QA/QC 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 multiple 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.

Implications for further research

Subsequent work will continue defining protocols and assay chemistries, add additional SNP and DIP markers, and further refine the packaging of components and reagents into a beta system. Developing an intuitive software and graphical user interface remains a high priority. Protocols and assays will further be streamlined for minimum complexity, time, and cost.

The successful commercialization of a sample-to-answer SNP and DIP-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.

V. REFERENCES

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VI. DISSEMINATION OF RESEARCH FINDINGS

Poster at the NIJ Conference, Arlington, VA, June 19, 2012,

Cooney CG, Sipes D, Thakore N, Holmberg R, and Belgrader P. (2012) A valveless PCR microarray flow cell that supports on-chip washing. *Biomed Microdevices*, 14:45-53.