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Improving Efficiency of a Small Forensic DNA Laboratory: Validation of Robotic Assays and Evaluation of Microcapillary Array Device

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Aim To present validation studies performed for the implementation of existing and new technologies to increase the efficiency in the forensic DNA Section of the Palm Beach County Sheriff's Office (PBSO) Crime Laboratory.

Methods Using federally funded grants, internal support, and an external Process Mapping Team, the PBSO collaborated with forensic vendors, universities, and other forensic laboratories to enhance DNA testing procedures, including validation of the DNA IQ™ magnetic bead extraction system, robotic DNA extraction using the BioMek2000, the ABI7000 Sequence Detection System, and is currently evaluating a micro Capillary Array Electrophoresis device.

Results The PBSO successfully validated and implemented both manual and automated Promega DNA IQ magnetic bead extractions system, which have increased DNA profile results from samples with low DNA template concentrations. The Beckman BioMek2000 DNA robotic workstation has been validated for blood, tissue, bone, hair, epithelial cells (touch evidence), and mixed stains such as semen. There has been a dramatic increase in the number of samples tested per case since implementation of the robotic extraction protocols. The validation of the ABI7000 real-time quantitative polymerase chain reaction (qPCR) technology and the single multiplex short tandem repeat (STR) PowerPlex16 BIO amplification system has provided both a time and a financial benefit. In addition, the qPCR system allows more accurate DNA concentration data and the PowerPlex 16 BIO multiplex generates DNA profiles data in half the time when compared to PowerPlex1.1 and PowerPlex2.1 STR systems. The PBSO's future efficiency requirements are being addressed through collaboration with the University of California at Berkeley and the Virginia Division of Forensic Science to validate microcapillary array electrophoresis instrumentation. Initial data demonstrated the electrophoresis of 96 samples in less than twenty minutes.

Conclusion The PBSO demonstrated, through the validation of more efficient extraction and quantification technology, an increase in the number of evidence samples tested using robotic/DNA IQ magnetic bead DNA extraction, a decrease in the number of negative samples amplified due to qPCR and implementation of a single multiplex amplification system. In addition, initial studies show the microcapillary array electrophoresis device (μCAE) evaluation results provide greater sensitivity and faster STR analysis output than current platforms.

The United States Bureau of Justice Statistics 2002 Census of Publicly Funded Forensic Crime Laboratories reported on the forensic backlog crisis in the Nation's more than 350 publicly

funded crime laboratories (1). It showed that demands on public forensic DNA laboratories have increased dramatically over the past five years, causing a tremendous strain on laboratory space, scientists, quality control/quality assurance administrators, training programs, and information system operators (1). The Palm Beach County Sheriff's Office (PBSO) Serology/DNA Section has conducted DNA analysis for over twelve years and has experienced the pressures associated with increased casework load. Since 2000, there has been a precipitous gap between the number of cases submitted and the number analyzed (46%). In order to address the issues of a burdened DNA program, the Section sought to identify and eliminate ineffective, laborious, and time-consuming tasks while preparing for future technologies. Validation of new methods can be a time consuming endeavor, but one that is an important and mandatory process for conducting analysis on forensic casework evidence (2,3). The Serology/DNA Section has evaluated and conducted several methodological validation studies in order to help alleviate the existing casework backlog.

The Serology/DNA Section of the PBSO used organic DNA extraction procedures for nearly ten years. This technique was time-consuming, labor intensive, used hazardous reagents, had the potential for PCR inhibitors to be extracted with the DNA, and did not allow for automation (4). In addition, even with concentration devices, organic extraction does not optimize the extraction of low template DNA samples. The Virginia Division of Forensic Science (VDFS) reported the use of a robotic workstation for caseworking laboratories using the Beckman Coulter BioMek2000 and showed the system increased the analysis of casework samples, saving time and labor (5). PBSO used the experience of the VDFS to validate and implement the BioMek2000® DNA robotic workstation and the addition of the Promega DNA IQ Extraction System (6,7).

Since the reports of an *in vitro* method of copying DNA, using a thermostable DNA polymerase (8-10), there have been extraordinary advancements in the biological and chemical sciences. Forensic Science has used the PCR to increase the potential for obtaining a DNA profile from biological samples that have been challenged or have low template DNA concentrations (11,12). The use of two multiplex systems, Promega PowerPlex1.1™ and PowerPlex2.1™, consisting of 15 STR genetic markers, was originally validated for casework, but this required amplification of twice the amount of DNA evidence, which was not always possible (13). The validation and implementation of a single multiplex STR system by the PBSO laboratory, PowerPlex 16 system, allows the amplification of 16 genetic markers, using approximately one nanogram of template DNA (14-16) in a single multiplex reaction. In addition to reducing DNA template amounts, analysis of evidentiary samples using this multiplex system has decreased the time necessary for interpretation of DNA profiles in half.

The FBI Standards state all unknown evidentiary samples must be quantified. The QuantiBlot® Human DNA Quantitation Kit (QB), which is based on the hybridization of a biotinylated oligonucleotide probe to an aliquot of an extracted DNA sample and subsequent detection by chemiluminescence, determines sample quantitation by a visual comparison of evidence samples to diluted DNA standards. The method is inherently inaccurate, can be aesthetically challenged, and time consuming. The goal of the PBSO laboratory was to implement a more objective method of quantification.

In 1999, the imaging system Hitachi CCDBIO became commercially available and was validated for use on casework evidence by the PBSO to decrease quantification analysis time. The QB protocol was conducted as per original protocol, but the probe-bound membrane was imaged and analyzed by a CCD camera (17). Although time was saved using an imaging system for quantification, QB negative samples were still amplified, adding additional time and cost to the DNA analysis procedure. The use of quantitative PCR has been used in many clinical and academic research protocols for DNA and RNA quantification (18-20). Quantitative PCR (qPCR) is based on the simple PCR premise that, through the use of fluorescently tagged primers, the accumulation of DNA during each PCR cycle can be detected, a threshold cycle determined, and the amount of DNA in a sample extrapolated. The advantages and issues associated with qPCR, such as primer and target DNA selection have previously been reported (18-20). In recent years the forensic community has also embraced qPCR technology specifically because of its sensitivity and large dynamic range (21-24). The PBSO validated the Ap-

plied Biosystems Sequence Detection System 7000 instrument and the Quantifiler Human and Y Quantitation Kits over a two month period demonstrating a fast and time-saving method to routinely use qPCR to quantify casework evidentiary samples, although the Y-Quantification system was not as reliable when compared to the human quantification system.

The forensic community is currently investigating the use of multicapillary electrophoresis devices in order to increase the throughput of forensic DNA samples (25). The PBSO is currently engaged in a collaborative effort with the University of California at Berkeley, Department of Chemistry and the Virginia Division of Forensic Science (VDFS) to increase DNA casework throughput by the evaluation of a microfluidic device for the rapid separation of STR fragments using a microcapillary array electrophoresis (μCAE) technology. The advantage of using a μCAE instrument over current multicapillary STR detection platforms includes the ability to utilize small sample volumes, thus conserving evidence, increased sensitivity, and greater throughput capabilities (26). Future studies will incorporate the use of energy-transfer cassettes bound to a primer for increased sensitivity (27).

The results of validation studies performed on a variety of techniques and methodologies that have greatly enhanced the capacity of a small forensic laboratory are presented. In addition, future approaches to enhance casework efficiency through microfluidic technology were addressed.

Material and Methods

Samples

Biological samples used for the validation of the instruments and reagents presented herein are either samples previously tested at the PBSO including non-probative, semen, and population samples, as well as sensitivity and mixed ratio samples generously provided by the National Institute of Standards and Technology (NIST).

Extraction

A reagent negative (N) and positive control (P) were analyzed with all extraction runs. DNA samples were extracted and purified using a single step organic extraction (2). In addition, samples were extracted using the Promega DNA IQ™ extraction kit (Promega, Madison, WI, USA). All reagents were supplied by the manufacturer in the DNA IQ kit except the dithiothreitol (Amresco Solon, OH, USA) and the Proteinase K (Amresco). Samples were placed in 1.5 mL dolphin microcentrifuge tubes. Each sample was extracted in 150 μL of Lysis Buffer. The samples were vortexed and centrifuged to force the cutting and lysis buffer to the bottom of the tube. Samples were placed at 57°C, 68°C, or 95°C, depending on the substrate composition for at least 30 minutes (if heat sensitive fabrics were used, e.g. polyester and nylon, extract without heating or at a lower temperature). Post-incubation, sample substrates were removed from the lysis buffer, placed in a Spin-Ex basket and centrifuged for 2 minutes at maximum speed. The spin basket and sample substrate were removed and stored. For semen stains, differential extractions were performed as follows: a portion of the semen stain was placed into a 1.5 mL microcentrifuge tube, containing 400 μL Tris/EDTA/NaCl, 25 μL 20% Sarkosyl, 75 μL H_2O, and 1 μL Proteinase K. Samples were incubated at 37°C for 2 hours, centrifuged for 5 minutes, the supernatant removed (the Female fraction) and the sperm pellet washed 3 times in sperm wash buffer. The samples were then loaded onto the 96 well plate. The BioMek2000 was prepared by initially conducting the Calibration and Alignment Test to verify the tips were touching in the correct plane, which ensured proper alignment with the deck. The deck was prepared with the appropriate materials and reagents as per manufacturer's instruction (6,7) and PBSO standard operating procedure. An assigned operator was responsible for selecting the Biomek sample method, distributing the tip boxes and test tubes on the deck and ensuring the circulating water bath was on. Once the deck set-up was completed, the operator executed the run by clicking on the "Start" button in the "Run" program. After the BioMek2000 run was completed, the operator capped and returned the 1.5 mL tubes, containing the final DNA elution to the appropriate analyst.

Quantification

Quantification of DNA samples was conducted using QuantiBlot (Applied BioSystems, Foster City CA, USA) with chemiluminescent detection by the Hitachi CCDBIO (Alameda, San Francisco, CA, USA) as per protocol. In some cases, Microcon 100's were used to concentrate

DNA samples. Prior to amplification, quantification of DNA samples was also conducted on the ABI 7000 Sequence Detection System.

Following the Calibration and Alignment Test, the BioMek 2000 was used to prepare the Quantifiler Human and Quantifiler Y (YPCR) reagents, standards, and samples as per protocol (28). Briefly, Quantifiler Human DNA standard was vortexed for 15 seconds to mix thoroughly followed by a pulse spin. Twenty microliters of Quantifiler Human DNA standard was loaded into the strip tube. The robot was programmed to automatically make the standard curve dilutions (Table 1) using the appropriate program based on the number of samples. The pipette boxes, 96 well plates, samples and a 1.5 mL test tube containing 40 µL of Tris EDTA was loaded onto the BioMek2000 deck. The following controls were run on each ABI 7000 run: Internal Positive Control (IPC), Optical Reaction Plate Control (OPC), where appropriate extraction Reagent Negative control (N), and Extraction reagent Positive Control (P) were also included. The BioMek2000 pipetted 23 µL of Master mix consisting of 10.5 µl of the Quantifiler Human Primer Mix or Y Human Male Primer Mix, 12.5 µL of the Quantifiler PCR Reaction Mix followed by 2 µL of sample and the standard curve. After the Biomek2000 loaded the extracted samples and standard curves into the 96 well plate containing the human or Y master mix, the 96-well plate was centrifuged at 3,700 rpm in a 96 well plate centrifuge for two minutes to remove any bubbles. The reaction plate was covered with an Optical Adhesive Cover, a Compression pad was placed over the Optical Adhesive Cover and the plate inserted into the ABI7000 heating block and the run initiated as per protocol. Quantitative PCR data was interpreted using the ABI7000 software program as per manufacturer's recommendations (28). To interpret the results, the data must be analyzed, the standard curve checked, and the internal positive control results assessed.

Table 1. Standard curve dilution for the ABI7000 Human Quantifiler System

Standard	Concentration (ng/µL)	Volumes	Dilution factor
A	50.0	20 uL [200 ng stock]+ 60 uL TE^{-4}/glycogen buffer	4×
B	16.7	20 uL [Std A]+ 40 uL TE^{-4}/glycogen buffer	3×
C	5.56	20 uL [Std B]+ 40 uL TE^{-4}/glycogen buffer	3×
D	1.85	20 uL [Std C]+ 40 uL TE^{-4}/glycogen buffer	3×
E	0.62	20 uL [Std D]+ 40 uL TE^{-4}/glycogen buffer	3×
F	0.21	20 uL [Std E]+ 40 uL TE^{-4}/glycogen buffer	3×
G	0.068	20 uL [Std F]+ 40 uL TE^{-4}/glycogen buffer	3×
H	0.023	20 uL [Std G]+ 40 uL TE^{-4}/glycogen buffer	3×

Amplification

All DNA samples were amplified using the Promega Corporation GenePrint PowerPlex 16BIO STR multiplex according to the manufacturer's recommendations (29) and as previously described (16). Typically 0.5 to 1 nanogram of DNA was used in the amplification reactions, unless otherwise specified.

Prior to polyacrylamide gel electrophoresis, PCR fragments were resolved using an Embitec 3% agarose gel to assess the extent of amplified DNA (EmbiTec, San Diego, CA, USA). The PCR reactions were electrophoresed in a 6% PAGE PLUS™ (Amresco) polyacrylamide gels. Conditions for electrophoresis were 6% PAGE PLUS at 60 Watts for 2 hours.

Short Tandem Repeat Detection

Detection of PCR products was performed using the Hitachi FMBIO® II Fluorescent Imaging System, a flat bed laser scanning instrument, and the FMBIO Analysis software. The color separation process was performed generally as described in the Promega PowerPlex 16 BIO Technical User's Manual (29). Allele sizing and designation, as well as determinations for optical density values were accomplished using the STaRCALL™ software program (29).

Results

There have been several technological and methodological transformations to the PBSO Serology/DNA laboratory over the past thirteen years. Validation of automated DNA extractions using DNA IQ and a robotic extraction station and quantification of DNA using real time methods, as well as initial evaluation results for a microcapillary device for STR allele separation and detection are presented.

Automated DNA Extraction

The PBSO used a single-phase organic DNA extraction procedure for nearly ten years. Promega's DNA IQ System uses a silica coated magnetic resin to bind DNA molecules that does

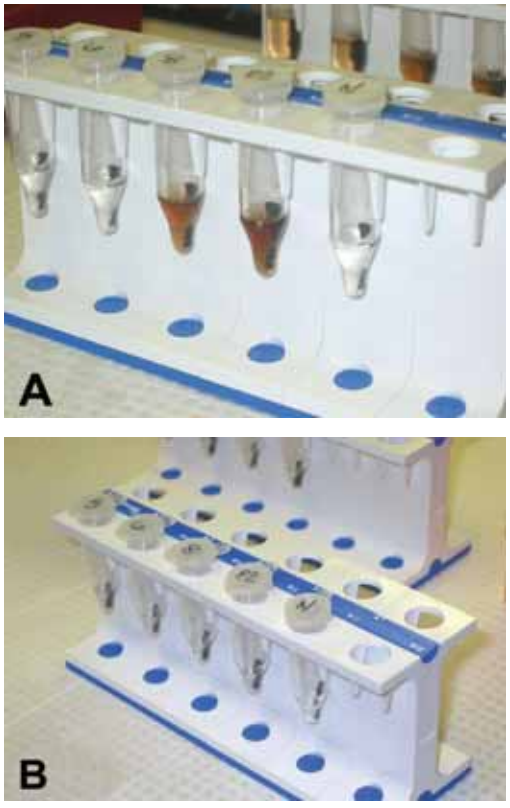

Figure 1. The manual DNA IQ™ Extraction System. **A.** Silica coated magnetic resin is added to samples previously incubated at 56°C to 96°C in DNA IQ™ Lysis Buffer. **B.** DNA binds to the magnetic resin, the resin is attracted to the magnetic stand where the lysis buffer is removed and the resin manually washed using DNA IQ™ Wash Buffer.

not require any centrifugation steps (7). The magnetic resin is pulled to the side of the tube in proximity to a magnet and the buffer can be removed without disrupting the magnetic resin pellet. Figure 1A shows the manual extraction protocol in which the sample has been incubated and magnetic beads added and the test tube placed on a magnetic stand. DNA binds to the beads and the beads are drawn to the magnet in the stand (Fig. 1B). Several wash and incubation steps are needed to obtain the extracted DNA. The manual extraction technique was determined to offer advantages over the organic extraction technique, but ultimately proved to be too time consuming and laborious to implement. As a direct result, the BioMek 2000 semi-automation DNA extraction station was validated.

The BioMek2000 robot is a simple liquid handling robot used for picking up tips individually

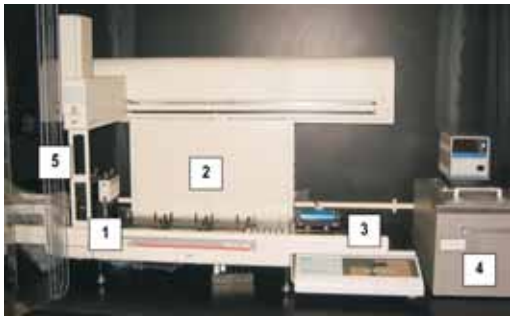

Figure 2. The Beckman Coulter BioMek® 2000 Laboratory Automation Workstation with modifications for DNA extraction using the DNA IQ™ Extraction System by Promega including: 1) Left Module (contains Tool Rack for holding MP200 pipetting tool- 8-channel, P200L pipetting tool-single channel, Gripper Tool, and other optional pipetting tools, 2) Base Module, 3) Right Module (contains shaking platform), 4) Circulating Water Bath (for heating Thermal Exchange Unit), and 5) Disposal Area.

or eight at a time, which allows for batching samples on a 96 well plate (Fig. 2). The deck of the BioMek2000 is shown in Figure 3. A gripper tool for picking up and moving plates around and a shaking platform for mixing samples are attached to the robot. The heat transfer block coupled to a circulating thermal exchange unit is mounted on a shaking platform and was used to elute the DNA from the DNA IQ™ magnetic resin. A circulating thermal exchange unit is heated via its attachment with tubing to an external water bath (Fig. 3). Aerosol resistant tips stored in specially designed boxes with enclosed wells may be re-used wherever possible.

There are limitations to the BioMek 2000 robot, such as a limited number of sensors

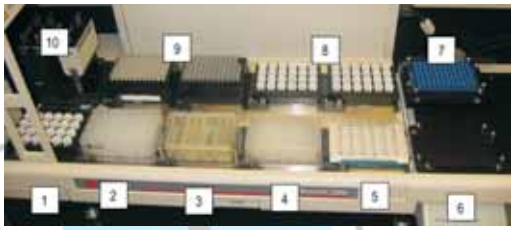

Figure 3. The deck of the Beckman Coulter Biomek® 2000 Laboratory Automation Workstation setup for extraction with the DNA IQ™ System including: 1) Microtube rack for holding 1.5 mL microcentrifuge tubes for the purified DNA, 2) Deep-well plate containing sample, 3) Reservoirs containing DNA IQ reagents, 4) Deep-well plate for waste disposal, 5) Magna Bot with Greiner Plate, 6) Shaking Platform with lab ware holder 7) Heat Transfer Block on Thermal Exchange Unit, 8) Microtube racks for holding 1.5 mL microcentrifuge tubes for the purified DNA, 9) 250 µL pipette tips, and 10) Tools.

and the inability to detect a clogged tip. Therefore, samples with large fragments of undigested material, such as tissue samples, must be centrifuged to pellet the debris prior to loading the lysate into a 96 well plate. PBSO is currently investigating the purchase of the BioMek NX System which is the next generation of robotic DNA workstations and will allow more versatility than the BioMek2000.

The VDFS initiated the original BioMek 2000 casework validation studies and collaborated with Promega Corporation to produce specialized software methods for the robot to integrate the DNA IQ extraction system (5,6). The methods are employed for the extraction of blood, epithelial cells, tissue, hair, and mixed biological samples. The PBSO has five major methods currently in use: the 16, 24, 40, 56, or 88 sample methods. The software program identifies the deck layout and calculates all reagent concentrations for each method (Fig. 4). All methods involve the use of deep well plates for the initial loading of samples, automated resin addition, and dispensation of the purified DNA into 1.5 mL micro-centrifuge tubes.

An important attribute of the DNA IQ System is the ability to purify small quantities of DNA efficiently. Approximately 100 ng of bound DNA will saturate the magnetic resin (6). Excess DNA is removed during the DNA purification process. Samples containing less than 10 ng of DNA are more efficiently extracted using the DNA IQ™ System (5). Since touch evidence is becoming more prevalent in criminal cases, the efficiency of extraction at these low template levels is critical to the success of obtaining a DNA profile, as well as the need to prevent contamination of samples (30,31).

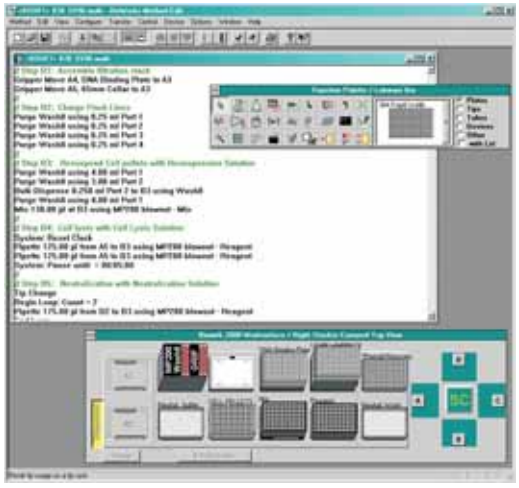

Figure 4. The BioMek2000 electronic visual desktop designed for programming of 16, 24, 40, 56, or 88 sample extraction methods. The software also provides a visual display of the correct layout of the tools, deck plates, and pipette tip.

The FBI National DNA Standards, "Section 8: Validation" (2,3) was used to validate the BioMek2000 robotic extraction protocol. The following is a summary of the approximately 400 samples that were analyzed for validation studies.

Non-probative Evidence. Five non-probative cases were evaluated for robotic extraction. PowerPlex 1.1 and/or PowerPlex 16 Bio STR data had previously been generated using organic extractions for blood, buccal swabs, and semen

Table 2. Non-probative casework samples; organic vs robotic extraction methods*

	Semen stain				Victim		Suspect	
Gene	NS robot	NS organic	SP robot	SP organic	robotic	organic	robotic	organic
FGA	23,26	23,26	21,24	21,24	23,26	23,26	19,22	19,22
TPOX	8,11	8,11	8,11	8,11	8,11	8,11	11,12	11,12
D8S1179	14(15)	14,15	14,(15)	14	14,15	14,15	13,14	13,14
vWA	17,18	17,18	17,18	17,18	17,18	17,18	13,16	13,16
AMELOGENIN	X	X(Y)	X(Y)	XY	X	X	XY	XY
PENTA E	12,13	12,13	7,(17)	7,17	12,13	12,13	13	13
D18S51	14,17	14,17	12,14,17,21	12,21	14,17	14,17	17,18	17,18
D21S11	28	INC	28,(33.2)	28,33.2	28,31.2	28,31.2	29,30	29,30
THO1	9.3	9.3	7,9.3	7,9.3	9.3	9.3	7,9	7,9
D3S1358	15,18	15,18	(15),18	18	15,18	15,18	14,15	14,15
PENTA D	NEG	13,14	INC	9,12	13,14	13,14	12,14	12,14
CSF1PO	11	11	11	11	11	11	12	12
D16S539	12	12	(12),13	13	12	12	11	11
D7S820	8,10,14	8,14	(8),10	10	8,14	8,14	10,11	10,11
D13S317	8,11,(12)	8,11,(12)	(8),11,(12)	11,12	8,11	8,11	11	11
D5S818	11	11	11	11	11	11	11,12	11,12

*Abbreviations: NS – non-sperm fraction; SP – sperm fraction; organic – organic DNA extractions with microcon concentration; robot – DNA IQ™ extractions on the BioMek2000; minor allele intensity is given in parentheses.

stains for a total of 20 samples. All results were comparable to the originally organically extracted samples. An example of the data generated from an adjudicated sexual assault case is shown in Table 2. Comparison of data generated from the robotic and manual organic extractions demonstrated shows that there was carryover of female DNA during the organic extraction but not during the robotic. Regardless, there were no foreign alleles to the victim and suspect (Table 2). In addition, the same conclusion reported for the case previously, ie, that the suspect is excluded as a contributor to the semen stain, was obtained.

Known Evidence. Known DNA profile samples were used throughout the robot validation and included dried blood, semen, buccal swabs, gum, epithelial cells, cigarette butts, and inheritance samples. There were no spurious or incorrect results for any of the known sample profiles. There are several instances in which the extraction of DNA from biological stains on certain substrates is difficult, such as blood on denim. One of the advantages of the DNA IQ extraction procedure is that it effectively removes inhibitors from samples (6,7). Figure 5A shows a QuantiBlot quantification film in which there is clearly DNA in buccal swab samples 8 and 9 (marked with one and two asterisks, respectively) yet sample 8 yielded no amplified products and #9 yielded partially amplified products (Fig. 5B). Attempts to dilute or add BSA to the amplification master mix also failed to yield a DNA profile. Extraction of the buccal swabs using the DNA IQ protocol provided amplified products (Fig. 5C) comprising a complete PowerPlex16 BIO DNA profile (data not shown).

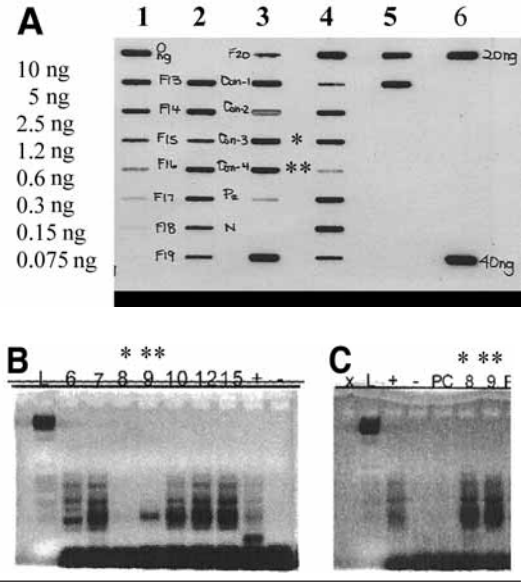

Figure 5. DNA IQ™ Extraction removes amplification inhibitors. **A.** Buccal swabs organically extracted, sample No 8 labeled Con-3 "*" and sample No 9 labeled Con-4 "**", yielded 1.18 ng/μL, and 3.62 ng/μL, respectively. **B.** Post-amplification agarose gel of organically extracted samples #8 and #9 with negative or partial amplification products and **C.** Post-amplification agarose gel of DNA IQ™ extracted samples #8 and #9 with positive amplification products.

Reproducibility

This standard was demonstrated by successful extraction of approximately 100 Reagent Positive Control (RCP2) for each set of extractions presented herein. Results of successful robotic extraction are based on a minimum of quantification results and a maximum of a complete predicted DNA profile. All resulting allele calls were in concordance with the known DNA profile at all 16 loci.

Table 3. Sensitivity study for the Biomek 2000 using neat to 1:64 semen dilutions

	Dilution ratio							
Gene	neat	1:1	1:2	1:4	1:8	1:16	1:32	1:64
FGA	20,23	20,23	20,23	20,23	20,23	20,23	20,23	20,23
TPOX	8,10	8,10	8,10	8,10	8,10	8,10	8,10	8,10
D8S1179	10	10	10	10	10	10	10	10
vWA	14,16	14,16	14,16	14,16	14,16	14,16	14,16	14,16
AMELOGENIN	XY	XY	XY	XY	XY	(X)Y	XY	XY
PENTA E	8,12	8,12	8,12	8,12	8,12	8,12	8,12	8,12
D18S51	10,12	10,12	10,12	10,12	10,12	10,12	10,12	10,12
D21S11	28	28	28	28	28	28	28	28
TH01	6,7	6,7	6,7	6,7	6,7	6,7	6,7	6,7
D3S1358	15,19	15,19	15,19	15,19	15,19	15,19	15,19	15,19
PENTA D	8,10	8,10	8,10	8,10	8,10	inc	8,10	8,10
CSF1PO	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12
D16S539	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12
D7S820	8,12	8,12	8,12	8,12	8,12	8,12	8,12	8,12
D13S317	11,12	11,12	11,12	11,12	11,12	11,12	11,12	11,12
D5S818	11,13	11,13	11,13	11,13	11,13	11,13	11,13	11,13
Total ng	11.5	8.7	5.4	3.15	1.65	0.95	0.4	0.25

Sensitivity

Whole blood and semen dilutions from neat to 1:64 were prepared as dried stains and all samples were extracted as per robotic protocol. Approximately 5 ×5 mm pieces were cut from each dilution stain for extraction. Results for both blood and semen dilutions indicate that the robotic extraction provided amplifiable DNA up to 1:64, with interpretable results (Table 3). Penta D at the 1:16 dilution was very weak and therefore interpreted as inconclusive. The blood dilutions showed full profiles obtained for 15 of the 16 genetic markers at all dilutions (data not shown). The Penta D locus results were either absent or uninterpretable. Full profiles for 1:16 semen dilutions were obtained with dropout of the Penta D. Note the newer lot number for the PowerPlex16 Bio kit was not as sensitive for the Penta D locus as in previous kits. Consequently, there have not been any difficulties obtaining Penta D with the current kit format. These same dilution series were compared with organically extracted samples with equivalent results (data not shown).

Mixture analysis. A series of mixture dilutions (female: male) including 100:0, 95:5, 90:10, 80:20; 66.7:323:3, 50:50 and the reverse for whole blood samples were prepared (Table 4). A total of 1 ng was amplified and 5 µl of the amplified product electrophoresed. A full DNA profile was evident for both female and male donors at all loci, except Penta D at 50:50. These results were comparable to the mixture analysis conducted using organic extractions. This imbalance is most likely due to a difference in the nucleated cell population of the male donor.

Semen mixture analysis. Fifteen total stains were prepared as follows: semen/vaginal (post-coital and mock samples), semen/blood and semen/buccal mixture samples were prepared. The differential extraction process was efficient in that both a female and male DNA profile was obtained in each of the fractions. The post-coital sample showed a major and minor profile in the non-sperm fraction in which alleles from both individuals were represented. The sperm fractions for all samples were of male origin only (data not shown).

Contamination

Two methods to test for potential robotic extraction contamination were performed.

Checkerboard validation study. This method depends on the distribution of samples across the 96-well plate in which each reagent negative control well is surrounded by an RCP2 sample. Initial results indicated the possible presence of low molecular weight amplified products in a negative well, as indicated by the post-amplification gel. However, vertical electrophoresis of the sample, followed by re-amplification and vertical electrophoresis of the reamplified sample did not indicate the presence of any amplified DNA. It is not clear what the source of the signal in the post-amplification sample was, but it could not be typed nor reproduced. No contamination was indicted in any of the negative samples, the selected RCP2 samples were positive for amplified products, and all alleles were confirmed.

Table 4. Female blood to male blood mixture ratio analysis using the BioMek2000® and DNA IQ™

	Detected alleles of genes																																							
Ratio	FGA				TPOX				D8				vWA				Amelogenin		Penta E				D18				D21				TH01				D3				Penta D				CSF1PO				D16S539				D7S820				D13S317				D5S818			
100:0	22	25			8	11			12	13			15	17			X		12	16			16	17			29				8				15				12				9		12		13				11		12		8		12		11		13	
95:5	22	25			8	(9)*			12	13			15	(16)			X		12	16			16	17			29				8			(9.3)	15		(18)		12				9		12		13				11		12		8		12		11		13	
					11								17																																												
90:10	22	25			8	11			12	13			15	(16)			X	(Y)	7		12		16	17			29				(6)		8		15		(18)		12				9		12		13				11		12		8		12		11		13	
									(16)				17						(16)												(9.3)																											
80:20	(20)	22			8	(9)			12	13			15	(16)			X	(Y)	7		12		16	17			29				(6)		8		15		18		INC				9		12		(12)		13		(8)		(10)		8		12		11		13	
	(25)				(11)				(16)				17						(14)		(16)										(9.3)																				11		12									
66.7:33.3	(20)	22			8	(9)			(12)	13			15	(16)			X	(Y)	7		12		16	17			(27)	29			(6)		8		15		18		INC				(9)		10		12		13		8		10		8		12		11		13	
	(25)				(11)				(16)				17						(14)		(16)										(9.3)												11		(12)						11		12									
50:50	(20)	22			8	9			(12)	13			(15)	16			X	(Y)	7		12		12	13			(27)	29			6		8		15		18		INC				(9)		10		12		13		(8)		10		8		12		11		13	
	(25)				11				(16)				17						(14)		(16)		(16)		(17)						9.3												11		(12)						(11)		(12)									
33.3:66.7	20	22			8	9			(12)	13			(15)	16			X	(Y)	7		(14)		12	13			(27)	29			6		8		(15)		18		INC				10		11		12		(13)		8		10		8		12		11		13	
	(25)								16				17										(16)		(17)						9.3																		(11)									
20:80	20	22			8	9			(12)	13			(15)	16			X	Y	7		(14)		12	13			(27)	29			6		(8)		(15)		18		INC				10		11		12		(13)		8		10		8		12		11		13	
	(25)								16				17																		9.3																											
10:90	20	22			8	9			(12)	13			(15)	16			X	Y	7		(14)		12	13			27	29			6		(8)		(15)		18		INC				10		11		12				8		10		8		12		11		13	
									16				17																		9.3																											
5:95	20	22			8	9			13	16			(15)	16			X	Y	7		14		12	13			27	29			6		9.3		18				INC				10		11		12				8		10		8		12		11		13	
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0:100	20	22			8	9			13	16			16	17			X	Y	7		14		12	13			27	29			6		9.3		18				INC				10		11		12				8		10		8		12		11		13	

*Parentheses – a minor or less intense allele. Abbrevations: INC – inconclusive.

Zebra-striped validation study. This method depends on the distribution of the RCP2 sample in five columns of the 96-well plate with alternating columns of reagent negative controls. No contamination was indicated in any of the negative samples, the two selected RCP2 samples were positive for amplified products, and all alleles were confirmed using PowerPlex16 BIO.

The entire BioMek2000 robotic validation process, training of analysts and generation of a caseworking manual was completed in six months.

DNA Quantification

The PBSO used the QuantiBlot DNA quantification system for eleven years and if a sample was negative for DNA template on the resultant film, the sample was still carried through STR amplification protocols, occasionally resulting in full DNA profile. If a sample were truly negative for amplifiable DNA template, an analyst would end up spending additional time and costs without achieving DNA profile results. Recently, PBSO validated the Applied BioSystems Sequence Detection System 7000 instrument and the Quantifiler Human and Y Quantitation Kits (32). The system utilizes quantitative PCR to measure the amount of amplifiable human or Y DNA in a sample. Each target specific assay consists of two primers for amplifying either higher primate DNA (Quantifiler) or higher primate male DNA (Quantifiler-Y) with one TaqMan MGB probe labeled with 5-FAM (5-Carboxy fluorescein) dye for detecting the amplified sequence (32). The TaqMan MGB probes contain a reporter dye linked to the 5′ end of the probe and a non-fluorescent quencher at the 3′ end of the probe. The 5′ nuclease activity, inherent in Taq polymerase, pre-extension step occurs during every cycle of the PCR amplification. The TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. AmpliTaq Gold DNA polymerase cleaves probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye resulting in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Therefore, non-specific amplification is not detected. A summary of the validation studies is as follows.

Plate Variation

A plate variation study was performed to ensure uniform amplification and fluorescent detection across the ABI 7000 heat block. 1.2 ng of cell line K562 was added to a Quantifiler primer and PCR master mix solution. The Biomek 2000 was utilized to load 25 μL of DNA containing master mix to every other column on two Optical Reaction Plates totaling 96 samples. The Optical Reaction Plates were prepared and quantified on two separate days. For each sample, it was verified that the IPC was above threshold and that the cycle thresholds were grouped together. The results of the plate variation study show that there was uniform amplification and fluorescent detection across the ABI 7000 heat block (data not shown). The mean cycle threshold for the 96 samples is 29.56 with a mean standard deviation of 0.16.

Precision

A precision study was performed to ensure that Quantifiler and Quantifiler Y gave similar DNA concentration values over a series of amplifications (Fig. 6). Ten dilutions of the cell-line 9,948 ranging from 10 ng/μL – 0.02 ng/μL were prepared. Three replicates of the dilution series were added to the Optical Reaction Plate and quantified with Quantifiler and Quantifiler Y. When comparing Quantifiler to Quantifiler-Y results, the diluted cell line 9,948 DNA samples gave similar cycle threshold concentration values for the same sample (data not shown). However, some of these mean concentration values were slightly higher than expected. In conjunction with the precision study, a standard curve study was also performed to measure the precision of the ABI 7000 assay utilizing the BioMek2000 to robotically prepare three-fold serial dilution with eight concentrations from the Human DNA Standard. Six replicates of the dilution series were added to the Optical Reaction Plate and quantified with Human Quantifiler (Table 5) and Quantifiler Y (Table 6). Quantification was performed by assigning one, two, or three of the dilution replicates loaded onto the Optical Reaction Plate as a standard(s). The diluted Human DNA standard samples gave similar concentration values for the same sample. The standard curve study illustrated that the precision of the ABI 7000 assay increases as the number of standard

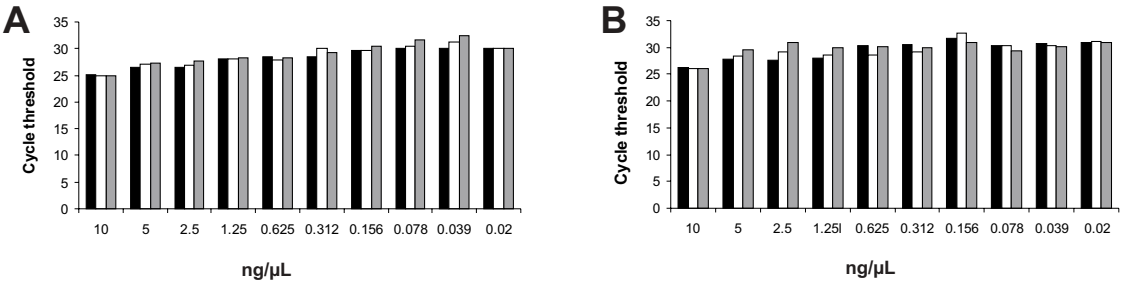

Figure 6. Human and Y ABI7000 Sequencer 9948 Precision data. Eight dilutions of 9948, ranging from 10 ng/µL-0.02 ng/µL were prepared. Three replicates of the dilution series were added to the Optical Reaction Plate and quantified with Quantifiler (**A**) and Quantifiler Y (**B**). Closed bars – replicate 1; open bars – replicate 2; grey bars – replicate 3.

curves run on a plate increases. The standard curve study further illustrates the precision of Biomek2000 to prepare and load the Human DNA standards is such that a reproducible slope range with a 98% or higher confidence level can be achieved with a minimum of one standard curve. To ensure accuracy, two standard curves will be run with each qPCR assay. In addition to illustrating increase precision with increasing replicates, the standard curve study also illustrates that precision of the Y standard curve replicates diminishes after the 5th dilution standard. Despite the diminished precision of Y standard curve replicates, it is possible to achieve a reproducible slope range with a 98% or higher confidence level, with a single standard curve. As a result, a two standard curve format has been implemented with each qPCR assay. In the event that one of the standard curves fail, running two standard curves will allow for an entire standard curve to be omitted during analysis if necessary.

The Quantifiler Y kit will be used as a screening tool to detect the possible presence of male DNA in a sample and not for quantification. DNA samples will be outsourced for Y-STR analysis when necessary (33,34).

Sensitivity

To determine the sensitivity of Quantifiler and Quantifiler Y real time PCR assays, ten di-

Table 5. Standard curve summaries for ABI7000 Human Quantifiler

Standard curve replicates	DNA	Mean CTs*	Standard deviation C^T	Mean DNA	Standard deviation DNA	%C.V.†
Three	50	23	0.091	46.89	2.844	6.07
	16.7	24.82667	0.019	13.73	0.173	1.26
	5.56	26.44333	0.08	4.63	0.251	5.42
	1.85	28.05	0.055	1.57	0.0574	3.66
	0.62	29.57667	0.12	0.563	0.0463	8.22
	0.21	31.22	0.134	0.186	0.017	9.14
	0.068	32.83667	0.141	0.0629	0.0059	9.40
	0.023	34.36	0.155	0.0226	0.0023	10.35
Two						
	50	23	0.091	46.68	2.824	6.05
	16.7	24.82667	0.019	13.71	0.172	1.25
	5.56	26.44333	0.08	4.64	0.251	5.41
	1.85	28.05	0.055	1.57	0.0575	3.66
	0.62	29.57667	0.12	0.567	0.0465	8.20
	0.21	31.22	0.134	0.188	0.0171	9.10
	0.068	32.83667	0.141	0.0637	0.006	9.39
	0.023	34.36	0.155	0.0229	0.0024	10.35
One						
	50	23	0.091	48.8	2.995	6.14
	16.7	24.82667	0.019	14.08	0.18	1.28
	5.56	26.44333	0.08	4.69	0.257	5.48
	1.85	28.05	0.055	1.57	0.0581	3.70
	0.62	29.57667	0.12	0.556	0.0463	8.33
	0.21	31.22	0.134	0.182	0.0168	9.23
	0.068	32.83667	0.141	0.0606	0.0058	9.50
	0.023	34.36	0.155	0.0215	0.0023	10.47

*Mean cycle thresholds (CTs) were determined using n=3.
†%C.V. equals the coefficient of variation; it was determined by dividing standard deviation DNA by mean DNA.

Table 6. Standard curve summaries for ABI7000 Quantifiler-Y

Standard curve replicates	DNA	Mean CTs*	Standard deviation C_T	Mean DNA	Standard deviation DNA	% C.V.†
Three	50	24	0.059	44.08	1.586	3.60
	16.7	25.6366	0.054	16.24	0.542	3.34
	5.56	27.33	0.062	5.75	0.221	3.84
	1.85	29.1433	0.135	1.89	0.154	8.15
	0.62	30.71	0.059	0.718	0.0265	3.69
	0.21	32.7833	0.403	0.205	0.0537	26.20
	0.068	34.5733	0.683	0.0704	0.0255	36.22
	0.023	36.2033	0.677	0.0259	0.0101	39.00
Two						
	50	24	0.059	41.91	1.478	3.52
	16.7	24.82667	0.019	13.71	0.172	1.25
	5.56	27.33	0.062	5.7	0.214	3.75
	1.85	29.1433	0.135	1.92	0.153	7.97
	0.62	30.71	0.059	0.743	0.0268	3.61
	0.21	32.7833	0.403	0.217	0.0557	25.67
	0.068	34.6033	0.683	0.0762	0.0271	35.56
	0.023	26.2033	0.677	0.0287	0.0109	37.98
One						
	50	24.01	0.059	40.32	1.382	3.43
	16.7	25.6366	0.054	15.58	0.495	3.18
	5.56	27.33	0.062	5.8	0.212	3.66
	1.85	29.1433	0.135	2.01	0.156	7.76
	0.62	20.71	0.059	0.799	0.028	3.50
	0.21	32.783	0.403	0.242	0.0602	24.88
	0.068	34.5733	0.683	0.0872	0.0303	34.75
	0.023	36.2033	0.677	0.0337	0.0125	37.09

*Mean CTs were determined using n=3.
†%C.V. equals the coefficient of variation and was determined by dividing standard deviation [DNA] by mean [DNA].

lutions of 9,948 ranging from 5 ng/μL-0.00975 ng/μL were prepared and quantified in triplicate with Quantifiler and Quantifiler Y (Table 7). Following quantification, one microliter of each dilution was amplified with PowerPlex 16BIO and electrophoresed on a 3% Embitec agarose post amplification gel. All samples that gave a positive post amplification result were electrophoresed on a 6% PAG vertical gel. A full DNA profile was detected for the unconcentrated 9,948 dilutions at a concentration of 0.312 ng/μL. Allelic dropout was observed in the unconcentrated 0.156 ng/μL and 0.078 ng/μL samples. The 0.039 ng/μL, 0.0195 ng/μL, and 0.00975 ng/μL dilutions were concentrated using a microcon-100 and a volume of 5 μL of Tris EDTA buffer was added to each concentrated sample. Five microliters of the concentrated 0.039 ng/μL, 0.0195 ng/μL, and 0.00975 ng/μL dilutions were amplified with PowerPlex 16BIO. A partial (13/16) but interpretable DNA profile was obtained for the concentrated, 0.0195 ng/μL and 0.039 ng/μL sample. An uninterpretable partial DNA profile was obtained for the concentrated 0.00975 ng/μL sample. The mean cycle threshold obtained for the 0.00975 ng/μL sample was 35. As a result, the cycle threshold cut off for a negative DNA sample was conservatively set at 36.

Table 7. Average ABI7000 quantification results (in ng/μL) for 9948 dilutions

Original DNA dilutions	QF DNA*	QFY DNA†
5	5.83	6.06
2.5	3.23	3.13
1.25	1.7	1.71
0.625	0.945	0.788
0.312	0.519	0.356
0.156	0.323	0.162
0.078	0.157	0.0543
0.039	0.0792	0.0193
0.0195	0.029	0.00436
0.00975	0.0246	0.00569

*Human Quantifiler quantification results, n=3.
†Quantifiler Y quantification results, n=3.

Reproducibility

Five single source oral swabs collected from male donors were quantified in triplicate on two different days with Quantifiler. The same five single source oral swabs collected from the male donors were quantified with Quantifiler Y in triplicate on the first day and duplicate on the second day. Similar concentration values for each single source sample in all replicates were obtained (data not shown). However, the Quantifiler Y concentrations were consistently lower than the concentrations reported by Quantifiler. The mean cycle threshold difference between Quantifiler and Quantifiler Y was approximately 2 cycles, as opposed to the expected one cycle difference. The deviance of the mean cycle threshold observed

with the Quantifiler Y kit can be attributed to the loss of precision in the last three standard curve dilutions.

In addition to the five single source male DNA samples, an aspermic sample and normal semen sample, and a single source male blood sample were quantified with Quantifiler and Quantifiler Y. All samples were amplified with PowerPlex 16BIO and electrophoresed on a 6% PAG. All the samples produced complete DNA profiles.

Mixture Study

Liquid blood samples from a male and female donor were used to create mixture samples with different male to female ratios (1:1, 1:2, 1:5, 1:10, and 1:20). The ratio dilutions were dried on a sterile cotton swab and approximately 25% of the swab was extracted. The mixture samples were quantified with Quantifiler and Quantifiler Y (Table 8). In the 1:1 sample, the Y-DNA concentration was 0.0831 µL and 5 µL was amplified. The male DNA minor profile was observed at 12/16 genetic markers. In the 1:2 sample, the male DNA profile was observed at 3/16 genetic markers. The male DNA was not observed in the 1:5, 1:10, or 1:20 mixture samples. The weak male profile obtained may be the result from the number of male cells present in the mixture or pipetting error in the mixture preparation.

The entire validation and training process took approximately six weeks.

Table 8. Average ABI7000 Human Quantification results (in ng/µL) for mixture set (n=3)

	Quantification results in male to female ratio				
[DNA]	1:1	1:2	1:5	1:10	1:20
Human	1.42	1.02	2.06	2.32	2.54
Y	0.105	0.0831	0.0735	0.0188	0.0618

Validation of Micro Capillary Array Electrophoresis Device

Dr Richard Mathies from the Department of Chemistry at the University of California at Berkeley in a collaborative effort with DNA forensic laboratories at the PBSO and VDFS has received a National Institute of Justice research grant (DNA Initiative Funding, 2004-2005) to evaluate the feasibility of using a microfabricated 96-channel radial capillary array electrophoresis (µCAE) microchannel plate-based platform to perform DNA profiling on casework evidence.

The microchannel plates (MCP) are fabricated in the University of California at Berkeley Microfabrication Laboratory. The MCP is a 150 mm diameter glass plate in which channels have been etched with hydrofluoric acid (HF) to form, after bonding to a second glass substrate, 200-µm wide by 25 µm deep capillaries stretching 17-18 cm from the twin-T 250-µm injector to the detection point (Fig. 7). Polyacrylamide is used to coat the channels, which prevents electro-osmotic flow. Separation of the STR products is conducted by electrophoresis through the channels that have been filled with Long Read LPA (Amersham, Piscataway, NJ, USA), a polyacrylamide used for conventional capillary electrophoresis. Upon injection of the DNA sample, the loaded MCP is placed on the Berkeley Rotary Confocal Fluorescence Scanner (Fig. 8). The plate is heated and a circular electrode array placed on top of the plate to make contact with the sample, waste, cathode, and anode reservoirs. After electrokinetic injection of the sample, the separation is conducted by

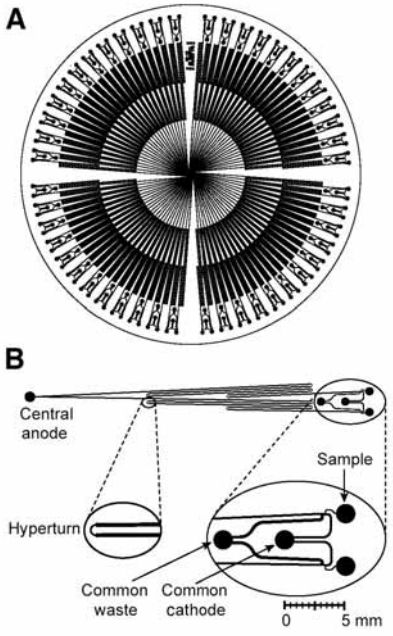

Figure 7. Microchannel plate. **A.** Microfabricated capillary array electrophoresis chip design. There are 48 doublet structures hydrofluoric acid etched on a 150-mm diameter glass wafer forming 96 lanes. **B.** The doublet capillaries allow for the injection of samples into two wells per doublet. A common cathode and waste well are present for each doublet. The sample is concentrated at the intersection of the sample arm and waste arm. The sample is electrophoresed down throughout the length of the channel towards the central anode.

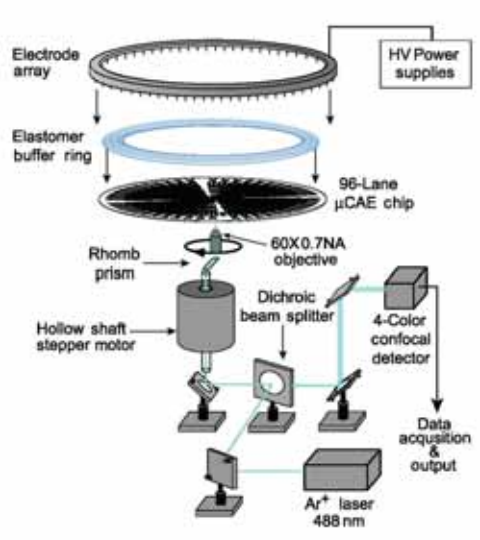

Figure 8. The rotary confocal fluorescence scanning system. An argon ion laser beam (488-nm) is passed through a dichroic beam splitter and is guided up the hollow shaft of a stepper motor. The beam is deflected 1 cm off the axis of rotation by a rhomb prism and focused by a 60× objective on the microfabricated 96-channel radial capillary array electrophoresis (µCAE) channels. The collected fluorescence travels back along the same path into a 4-color confocal detector. A polydimethylsiloxane (PDMS) elastomer ring on top of the µCAE plate is used to create a continuous buffer reservoir for the cathode and waste wells. An electrode array is used to supply voltage to various reservoirs.

application of +2500 V at the anode reservoir, +0 V at the cathode reservoir, +180 V to the sample reservoir and +200 V to the waste reservoirs. The laser-excited rotary confocal fluorescent scanner is then used to detect the tagged STR products. Data analysis is collected and stored as a data-appended text (DAT) files and stored in pre-named run files. Raw data is converted to the proper format and analyzed using the MegaBACE Fragment Profiler and Genetic Profiler software programs.

The performance evaluation of the µCAE instrument is being conducted using the FBI Standards (2,3) as a model and includes precision, sensitivity, reproducibility, mixture, and non-probative testing. In addition, additional testing will be conducted to determine if there is any carryover or contamination issue within the µCAE process.

To date, the National Institute of Science and Technology (NIST) has provided DNA samples to test sensitivity and mixture resolution, as well as additional samples including single source and non-probative samples (manuscript in progress). DNA samples are amplified at the PBSO laboratory using Promega PowerPlex 16 and Applied BioSystems Profiler Plus STR multiplexes, as per manufacturer's recommendations. The samples are then shipped overnight to the VDFS and the Berkeley laboratories for analysis on the Applied

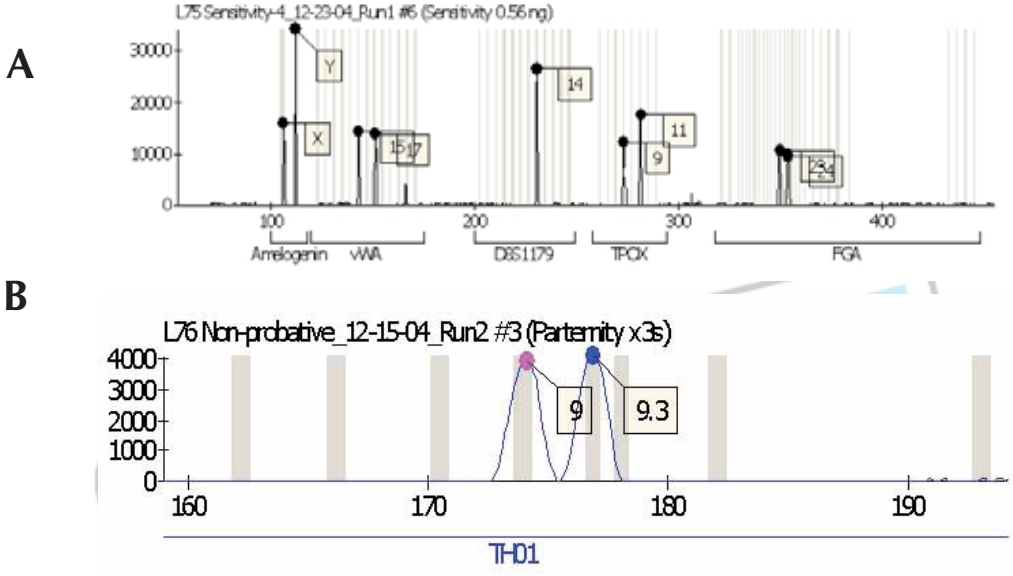

Figure 9. Microcapillary electrophoresis of single source DNA sample amplified with PowerPlex16 BIO, analyzed on the microcapillary device and data interpreted using the MegaBACE program. **A.** Example of the Rhodamine Red-X fluorophore channel for a population sample. **B.** Resolution of the 9.3/10 THO1 alleles in a non-probative DNA sample.

BioSystems CE310 Genetic Analyzer and the μCAE instrument, respectively. Results from the analysis are sent to PBSO and VDFS for analysis and interpretation of the electrophoresed samples. Initial evaluation results have shown that the μCAE device separates STR fragments in a fraction of the time needed for flatbed or conventional capillary devices such the CE310 instrument. A typical amplified product separation occurs in under 25 minutes for up to 96 samples. The μCAE readily resolves profiles (Fig. 9A) STR allelic ladders and single base pair differences such as the THO1 9.3/10 alleles (Fig. 9B).

An additional important advantage is that future microfabricated chips will contain integrated sample preparation methodologies such as micro PCR devices, which will further enhance the speed, improve reliability, and reduce cost and labor for analysis of forensic DNA case (26). It is expected that in conjunction with extraction robotics and qPCR protocols, the use of the μCAE instrument will greatly enhance the efficiency of forensic DNA laboratories.

Conclusion

Many high-throughput forensic and medical DNA laboratories have implemented protocols using robotics and microchip devices. The PBSO has demonstrated that many of these technologies may be implemented in a small forensic DNA laboratory. The result is a remarkable increase in the efficiency of the laboratory to conduct DNA analysis on an ever-increasing caseload in a timely manner without compromising quality. It is important to note that many laboratories will share data or train individuals from other laboratories in order to help with the transition to a more efficient laboratory. A preliminary evaluation of a next-generation microfabricated capillary array electrophoresis microchannel plate technology is ongoing and currently appears to be promising for further improvements in forensic speed, cost, and quality.

Acknowledgements

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