Final Report

Microfabricated Capillary Array Electrophoresis Genetic Analyzers for Forensic Short Tandem Repeat DNA Profiling

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Contents

ABSTRACT

The overall goal of this project was to develop faster, more reliable, higher throughput, more sensitive and more thoroughly integrated technologies for forensic STR identification. Inspiration for this effort comes from technologies developed over the past 15 years as a part of the human genome project. These technologies include the use of more sensitive energy-transfer fluorescent dye labels, the development of microfabricated capillary array electrophoresis separation and fluorescence detection systems, and the integration of sample clean-up and PCR amplification with the separation structures. With this motivation we have: (i) developed a high-throughput 96 channel microfabricated capillary array electrophoresis wafer system and confocal rotary fluorescence scanner and demonstrated its effectiveness for performing forensic STR separations with conventional commercial multiplex kits; (ii) transitioned this new microchip forensic analysis system to the forensic laboratory at the Department of Forensic Science at Virginia and established its practicality for routine forensic analysis (iii) developed a modified version of the PowerPlex 16 STR kit that employs energy-transfer or ET dye labeling for enhanced (2-8-fold) signal strengths for improved STR fragment separation and detection; (iv) developed a portable single channel forensic analysis instrument that includes the steps of PCR amplification, sample and standard injection, separation and fluorescence detection in a single integrated chip and instrument. Following validation we have performed field trials to demonstrate the ability of this system to perform STR analysis in the field producing real-time forensic identification; (v) performed initial feasibility studies of using gel-supported oligonucleotide capture matrices for the concentration and purification of amplified STR products for enhanced sensitivity capillary electrophoresis injection and analysis; and (vi) initiated the development of a table top version of our 96 channel rotary scanner and chip system that will facilitate the wide utilization of this new technology by laboratories desiring both low and high throughput.

Summary of Research Findings and Conclusions

1. *Evaluation of μCAE performance with commercial STR typing systems*

A 96-channel microfabricated capillary array electrophoresis (μCAE) system as shown in Figure 1 was evaluated for forensic short tandem repeat (STR) typing using PowerPlex 16® and AmpFℓSTR® Profiler Plus® multiplex PCR systems. Performance evaluated included analysis speed, fragment resolution, profile concordance, mixture allele discrimination, sensitivity and real-world forensic sample typing capability. Results of this work and the technical details were disseminated in the *Journal of Forensic Sciences.*[1]

Speed, Resolution & Concordance: The high-throughput μCAE system produced 96 high-speed (< 30 min) parallel sample separations with single-base resolution based on the 9.3/10 THO1 alleles in the PowerPlex 16 allelic ladder. Forty-eight single-source samples previously analyzed by PBSO were accurately typed, as confirmed on an ABI Prism 310 and/or the Hitachi FMBIO II.

Mixture study: STR samples consisting of male and female DNA at the ratios of 10:0, 9:1, 3:1, 3:2, 2:3, 1:3, 1:9 and 0:10 were analyzed. The 3:1 and 1:3 samples are the lowest (highest) ratios in which all minor components were detected and reliably typed.

Sensitivity study: The sensitivity of the μCAE system was assessed using PowerPlex 16 and Profiler Plus samples amplified with serially diluted DNA templates (from 22 to 0.0054 ng). The instrument produced full profiles from sample DNA down to 0.17 ng, yielding 100% of the expected profiles for both tying kits; a threshold similar to that found for the ABI 310.

Forensic DNA sample typing: Seventeen non-probative samples were correctly typed from case evidence previously processed and analyzed by PBSO using both the PowerPlex 16 and Profiler Plus systems. The DNA extracts were collected from a variety of common stain sources encountered in forensic analysis, including semen, saliva, single and mixed blood stains from sexual assault, paternity, burglary, armed robbery as well as homicide cases. The DNA data obtained using the μCAE system for the less complicated paternity and single-source bloodstain case samples produced full profiles

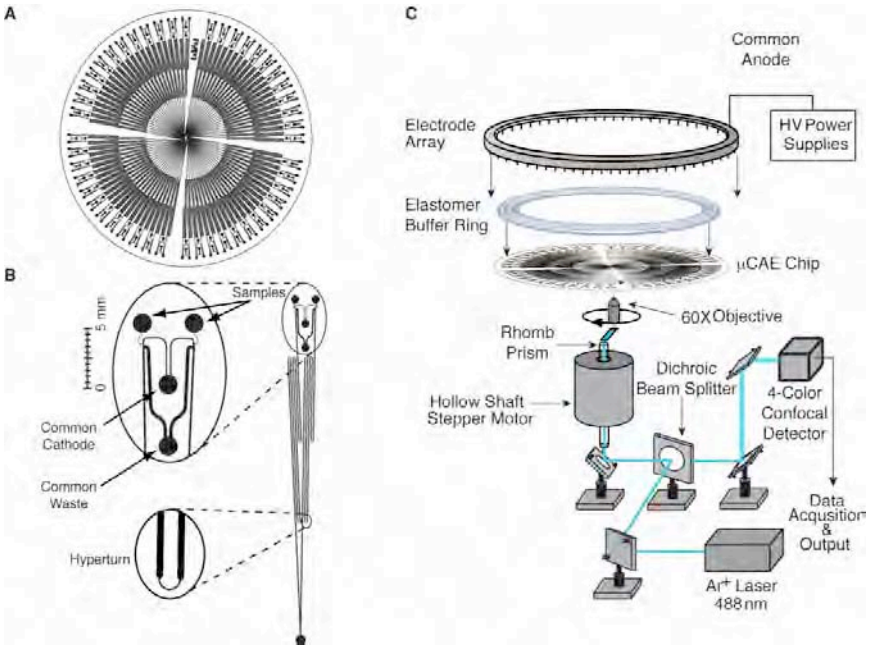

Figure 1. (A) Layout of the 96-lane μCAE chip. The 96 adjacent microchannels are grouped into 48 doublets on a 150-mm diameter glass wafer. (B) Each doublet contains two cross-injector structures with two sample wells that share a common cathode and waste wells. (C) The rotary confocal fluorescence scanning system consists of a 488-nm argon ion laser beam which is passed through a dichroic beam splitter and directed up the hollow shaft of a stepper motor. The emitted fluorescence travels back along the same path into a 4-color confocal detector.

that matched with the previously reported case results. For the sexual assault DNA samples, both μCAE and ABI 310 profile results for the sperm-fraction obtained from the vaginal-swab semen agreed with the original report analysis - the DNA profile did not match the suspect's DNA profile. However, the μCAE system was able to discern additional alleles in the nonsperm fraction of the semen stain, consistent with the semen contributor that were not callable previously using the FMBIO II. For the bloodstain mixture collected from a knife blade and another from a sandal in a homicide, the μCAE profile results were identical to the original report as well as to the ABI 310 analyses, even at loci with imbalanced peak heights indicating major and minor contributors.

This work successfully demonstrated that current standard commercial forensic typing kits can be effectively applied to our μCAE system with no modification - a significant step towards the ultimate goal of developing of a completely integrated forensic STR typing microsystem.

2. Development and validation of a prototype μCAE forensic scanner for VDFS

A prototype μCAE chip scanner system was successfully installed in VDFS and validated by VDFS forensic scientists in July 2007. The work on validation has been submitted to the *Journal of Forensic Sciences*[2]. The system consists of a four-color microchannel plate (MCP) scanner (Fig. 2A), a gel/sample loader, and a chip cleaning station (Fig. 2B and 2C). There were three major stages in achieving this goal.

In the first stage, we performed initial testing on the scanning system for STR typing operation, troubleshot and identified the needs of additional software for MCP plate focusing on the stage as well as electrical current monitoring functions. We also tested the gel/sample loader and chip cleaning station for their ability to perform the appropriate functions as expected. In December 2005, forensic scientists Susan Greenspoon and Amy McGuckian visited Berkeley for their first training on the system.

The second stage marked the transfer of the MCP system outside of a research laboratory setting to a forensic one. The MCP system was shipped and installed at VDFS in April 2006 followed with fine tuning and minor adjustments of the operation procedures.

The final stage marked the mastery of the MCP system by VDFS forensic scientists and its validation for forensic STR typing. Standard validation assays performed include resolution measurements, concordance, sensitivity and mixture studies as well as non-probative and Y-STR sample typing. We have also examined the success rate of the 96-lane system and investigated alternative microchannel coating methods for more user-friendly operation. STR data produced on both the μCAE device and the ABI 310 were compared. The data produced from the μCAE device were sent to PBSO for comparison with previous DNA profiling results for the cases as well.

Figure 2. Pictures of the MCP system installed at VDFS consisting of (A) a four-color confocal scanner, (B) a gels/sample loader and (C) a microchip washing station.

Operation of the μCAE device followed the procedures outlined by Yeung *et al.*[1] For μCAE chips coated using the modified Hjerten procedure,[3] a fresh coating was applied every two weeks (S. Yeung, personal communication). For μCAE chips coated using the polyDuramide (pDuramide) dynamic coating polymer,[4] we followed procedures outlined in Ref. 4. A fresh coating was applied every 5 days. The following highlights the findings and technical details associated with each study.

Precision, Resolution, Success Rate & Concordance: Precision results obtained by separating PowerPlex 16 allelic ladder samples on the μCAE instrument were compared to those obtained on ABI 310 at VDFS as well as on commercial multi-capillary instruments previously. The single capillary ABI 310 at VDFS displayed better precision than the μCAE as well as data previously obtained at Berkeley. However, the precision of μCAE is essentially equivalent to commercial CAE systems (ABI 3100, 3700 and MegaBACE 100).

Resolution measurements were obtained based on the TH01, CSF1PO, TPOX & Amelogenin loci in PowerPlex 16 allelic ladder using methods detailed in Buel *et al.*[5] Briefly, resolution results obtained using μCAE were similar to those reported by various sources for ABI 310 as well as those measured for μCAE by Yeung *et al.*[1] using the TH01 locus. Resolution measurements for both Hjerten and pDuramide coating methods were nearly identical and were similar to those produced for the μCAE at Berkeley. Peak morphology and separation between the 9.3 and 10 alleles of the TH01 allelic ladder were virtually identical between the two coating procedures and similar to that produced by ABI 310. In addition, the pDuramide coated μCAE microchips (8 runs) demonstrated a greater number of open capillaries (~ 20% more) than the modified Hjerten coated microchips (7 runs).

For the concordance study, 47 single-source DNA samples were extracted and purified from buccal swabs and dried blood cards by VDFS. Full concordance was obtained for all 47 DNA profiles as confirmed using the ABI 310 and by the VDFS staff DNA index.

Sensitivity & mixture studies: The DNA samples were purified from either buccal swabs or tissue samples as defined.[5] The sensitivity study was performed on microchips coated with the Hjerten and pDuramide dynamic coating procedure using a sensitivity series provided by NIST and VDFS. As demonstrated in Figure 3, the results showed that the sensitivity of the μCAE device was not affected by the coating and comparable to that reported for commercial CE instruments[6-8] and to that reported by Yeung *et al.*[1]

In mixture study, PowerPlex 16 samples amplified using mixed DNA samples prepared by VDFS at the ratios of 1:0, 9:1, 3:1, 3:2, 2:3, 1:3, 1:9 & 0:1 were analyzed using microchip coated with pDuramide. The majority of minor contributor alleles were detected at the 3:1 and 1:3 ratio samples. While all minor components were detected in these samples previously in Yeung *et al.*[1], alleles below threshold were observed for allele 12 at D3S for the 3:1 samples, as for allele 9 at TH01 and allele 11 at Penta E for the 1:3 sample. These differences were possibly due to the differences in methods used in estimating DNA concentration by VDFS and by NIST in Yeung *et al.*[1] For both the 9:1 and 1:9 samples, minor contributor alleles were above the peak threshold at many loci, consistent with other reports.[1,7-9]

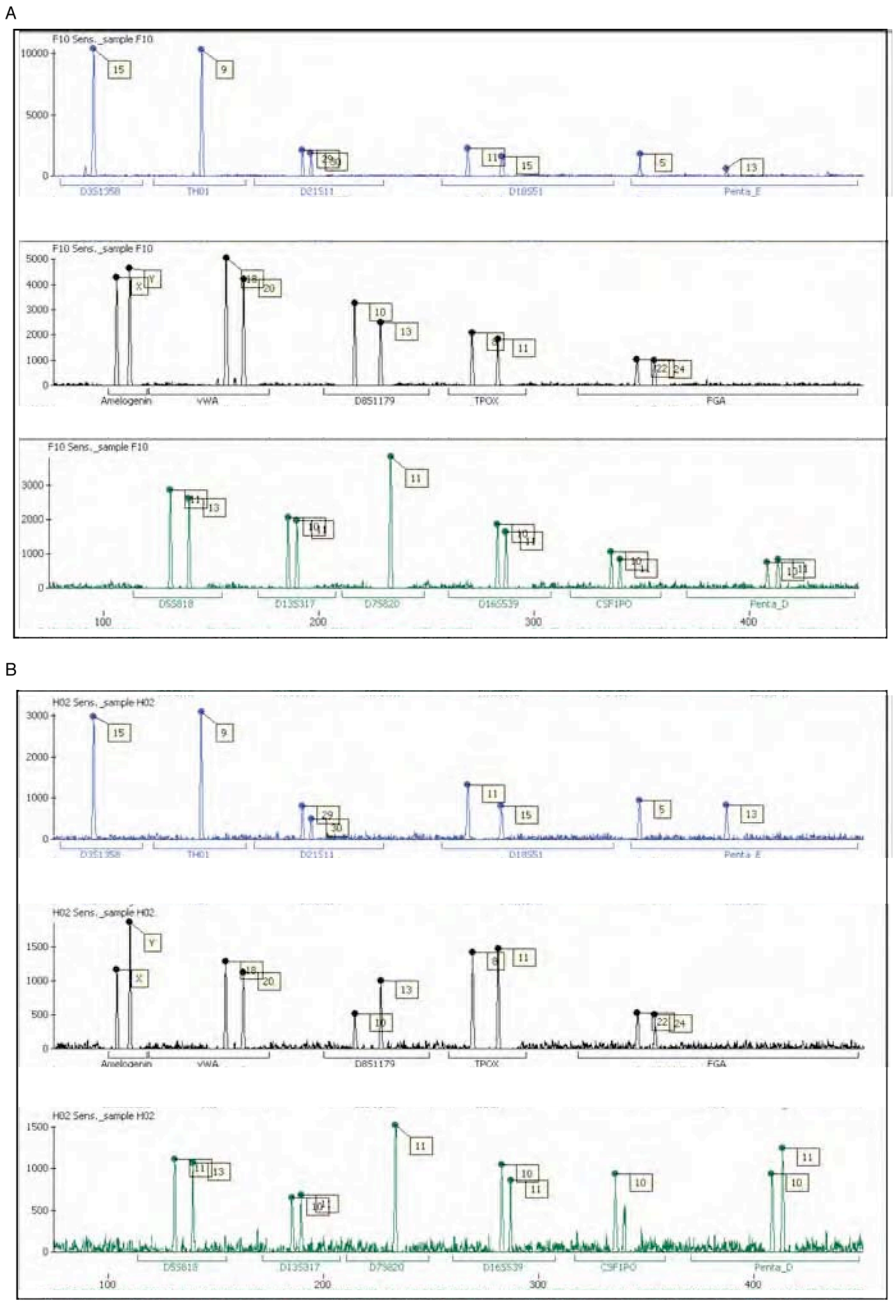

Figure 3. MicroCAE PowerPlex 16 profiles of sensitivity study samples using a single-source male DNA sample amplified with (A) 2 ng of input DNA and (B) 125 pg of input DNA. (Top: Fluorescein channel, Middle: TMR channel, Bottom: JOE channel.)

Non-probative analyses: PowerPlex 16 samples of 19 non-probative DNA extracts from five cases were prepared. All DNA samples were quantified at PBSO. Although DNA profiling was originally done using various PCR-based human identification kits, 100% concordance was obtained for μCAE data compared to the original case reports (C. Crouse, personal observations).

The successful transfer the prototype μCAE system to a forensic laboratory setting and its application to actual forensic STR typing samples without significant modifications further demonstrate its functionality and attests to ease of use of the final instrument and protocol. A review paper on this topic and the vision of integrated microchip technology for forensics will be published in Promega's *Profiles in DNA* to inform the forensic community.[10]

3. Development of energy-transfer (ET) cassette labeled multiplex typing systems

A 16-plex STR typing system has been developed[11] with improved energy-transfer fluorescent dye cassette labels using primer sequences in the PowerPlex 16 kit. This kit was chosen because of its readily available sequence information in the public domain.[7] To maintain similar dye-induced mobility shifts, FAM labeled loci were replaced with FAM-FAM cassette, JOE with a FAM-R6G cassettes, and TMR with FAM-TAMRA cassettes. Each ET primer was subject to systematic monoplex PCR and μCAE evaluations to ensure it was yielding the expected increase fluorescent signals before it was included in the multiplex. Before multiplex construction, we also looked at the effective annealing temperatures of three cassette labeled primers to show that ET-primers behaved like the single-dye primers. We then compared the performance our ET 16-plex with PowerPlex 16 to evaluate its ability to produce higher STR amplicon fluorescence signals from low-level DNA, to amplify DNA templates at reduced PCR cycle counts, and to perform typing on challenging DNA samples extracted from variety of forensic evidence. All μCAE operation and analysis procedures follow Yeung *et al.*[1] except for microchannel coating procedure which follows a pDuramide coating procedure. The findings and technical details are summarized below for each study.

Annealing temperature study: The study characterized five annealing temperatures of energy-transfer (ET) cassette-labeled primers for three loci, (TH01, Penta D and vWA); each primer was coupled to a different ET-cassette label and compared in parallel to the corresponding single-dye labeled primers. The results of this study show that the ET-primers and single-dye primers follow similar annealing trends. It is

observed that 60 ^{o}C is the most effective annealing temperature for all three ET-cassette labels.

Increased fluorescent signals: Equimolar ET-cassette labeled STR primers were electrophoretically separated together with the corresponding single fluorescent-dye labeled primers. ET-cassette labeled primers yield 1.5–8X higher fluorescence intensities. To examine the effects of thermal cycling on ET-labeled amplicon signals, amplified PCR products were produced with both types of dye labeled primers on three STR loci under identical conditions. The amplified ET labeled PCR products displayed the expected increase in fluorescence intensities. These results confirm that higher fluorescence-signal PCR products can be achieved using ET-cassette labeled primers under the same PCR conditions as conventional single fluorescent-dye primers.

The ET 16-plex typing system was constructed first using the same primer concentrations in PowerPlex 16 (Promega, personal communication) followed with subsequent adjustments of primer concentrations by typing of a single-source DNA sample, A-18 obtained from Promega. The final ET 16-plex primer concentrations used were either the same or lower than those in PowerPlex 16 with equal forward and reverse primer concentrations (except for vWA which is 2X the concentration of the commercial system for ease of balancing). A 10X single-dye primer mix was also prepared using the same primer concentrations for in-house control.

Representative A-18 profiles generated with both ET 16-plex and the PowerPlex 16 kit are shown in Figure 4. ET-cassette labeled STR alleles yielded 1.6–9X higher fluorescence intensities than single-dye labeled alleles amplified with PowerPlex 16. Figure 5 illustrates the increased signal-to-noise (S/N) ratios of 2–6.5X for FAM-FAM loci, 1.6–6.6X for FAM-R6G loci and 2–8.7X for FAM-TMR loci compared to the corresponding single-dye labeled fragments. The increased signal intensity at an STR locus is the averaged ratio of the ET-allele S/N to the single-dye labeled allele based on two data sets (four alleles for heterozygotes and two alleles for homozygotes) from identical PCR reactions analyzed within the same μCAE run.

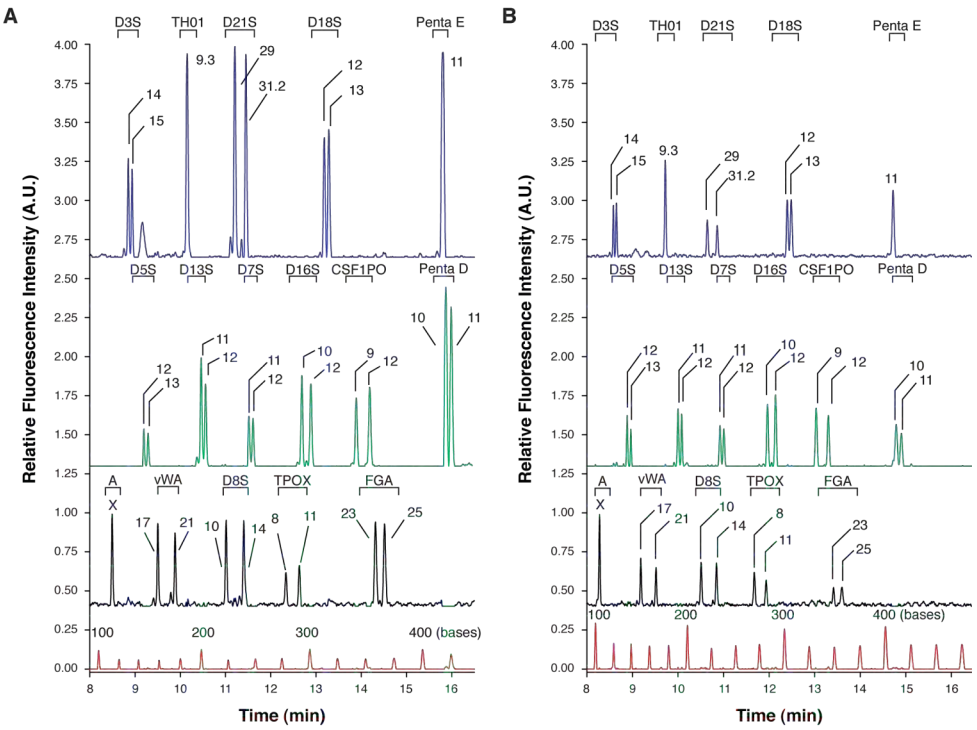

Figure 4. Profiles of 0.5 ng of A-18 DNA amplified with (A) ET 16-plex and (B) PowerPlex 16.

Sensitivity study: The sensitivity of the ET 16-plex STR typing system was assessed and compared to PowerPlex 16 using samples amplified from a dilution series of A-18 DNA. Figure 6 plots the percent allele detection from 0.25 to 0.0039 ng of DNA template. All 29 expected STR alleles (defined as S/N ≥ 3) are achieved with the 62.5-pg samples amplified with the ET 16-plex. At the same DNA input, only 79% and 72% of the profile was successfully typed from the PowerPlex 16 and the in-house SD 16-plex samples, respectively. With only 31.3 pg of DNA we could still detect ~97% of

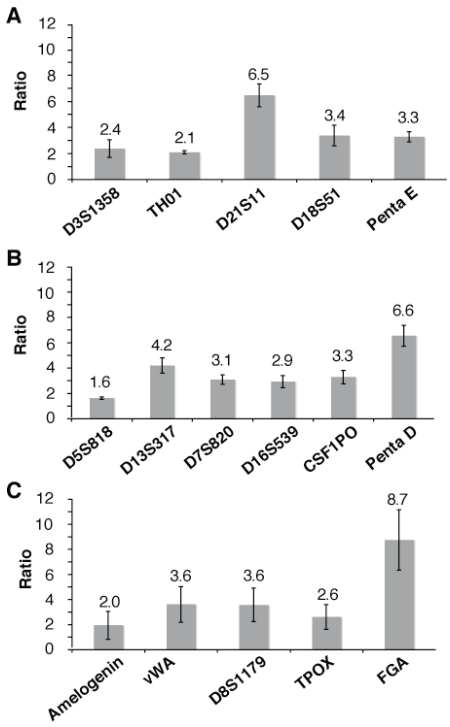

Figure 5. Average fluorescence intensity ratios of ET labels versus single-dye labels at each STR locus. (A) 2–6.5X for FAM-FAM labeled loci, (B) 1.6–6.6X for FAM-R6G loci and (C) 2–8.7X for FAM-TMR loci.

the profile using the ET 16-plex. This increase in sensitivity over the single fluorescent-dye labeled multiplex systems demonstrates the capability of ET primers to achieve higher signals or the same signals with less template DNA. This achievement should advance low-copy number (LCN) typing[6,12] by eliminating the need for increased thermal cycles which frequently results in non-specific amplification. At the same time, the reduced DNA requirement for a meaningful DNA profile translates into a higher STR typing success rate and reduces the cost and delay caused by repeat typing.

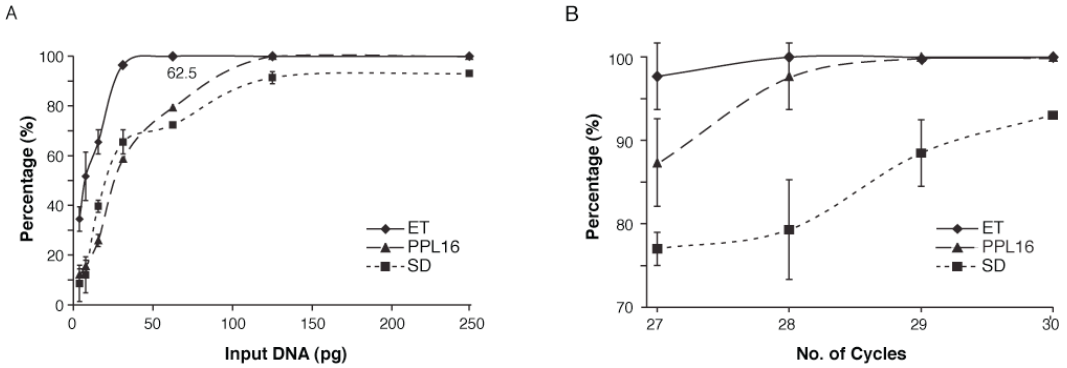

Figure 6. Percentage of full STR profiles obtained for DNA samples amplified with ET 16-plex, single-dye 16-plex and PowerPlex 16 as a function of (A) amount of input DNA and (B) the number of PCR cycles.

Reduced PCR Cycle Study: The traditional approach for performing STR typing of poor-quality DNA is to increase the number of PCR cycles, but this can evoke false amplification or allele drop-in.[6, 12-14] With the increased sensitivity of ET labels, we demonstrated the possibility of reducing the total number of PCR cycles to speed analyses and reduce non-specific background. We performed experiments to type 0.5 ng of DNA template at 30, 29, 28 and 27 cycles. Figure 6B illustrates that full profiles were obtained using the ET 16-plex with as few as 28 cycles and a 97% profile was obtained at 27 cycles. The higher performance of the ET 16-plex was further exhibited when comparing the 27-cycle PCR samples (98% vs. 87% profile). The results of this study demonstrated the potential of using ET-cassette labeled primers to achieve typing of compromised DNA samples without raising the number of PCR cycles, thereby reducing analysis time and complication in DNA profile interpretation.

Real World Samples Typing: To explore the potential of using ET cassette labeled primers to perform analyses on casework samples, we have typed 6 difficult forensic DNA samples from case evidence previously processed by PBSO. The sexual

assault case DNA samples were extracted from buccal swabs, semen swab from underwear (both sperm and non-sperm fractions), whole blood and blood swabs from a towel. All DNA samples were amplified using the ET 16-plex and PowerPlex 16 systems under the same conditions and it was shown that the ET 16-plex produced equivalent or better results in all cases.

4. *Development of single channel integrated PCR-CE analysis system on a portable instrument:* We have successfully developed and evaluated a portable forensic analysis system consisting of a microfluidic device for amplification and separation of STR fragments together with an instrument that contains 4-color confocal laser fluorescence detection and all the necessary optical and electronic components for chip operation. To explore the forensic applications of this portable system, a 4-plex mini-Y chromosome STR typing kit was first developed and successfully performed on the microsystem with a 90-min analysis time. The performance of this system for forensic STR typing was evaluated on sensitivity, real-case sample and mixture analyses. A field demonstration of the real-time DNA typing was successfully performed. Results of this work and the technical details were disseminated in the *Analytical Chemistry.*[15] To further realize a practical on-site STR typing, a 9-plex autosomal STR typing system was developed and optimized. We are now exploring the feasibility of using our portable microsystem to perform real-time DNA analysis at a mock crime scene in collaboration with PBSO.

Microdevice design and fabrication: The 1st generation microdevice we developed for 4-plex mini-Y STR typing contains a 160-nL PCR chamber with an integrated heater and a four-point resistance temperature detector (RTD) for PCR amplification and a 7-cm-long CE separation channel for CE separation (Figure 7A). To extend the microdevice to autosomal STR typing, a 2nd generation of the PCR-CE microdevice with a co-injection structure has been constructed to achieve sizing calibration. As shown in Figure 7C, a 20-μm wide microchannel connected to a sizing standard reservoir was added to the cross injector. The width ratio of the co-injection channel to the PCR sample channel was optimized to achieve balanced injection of PCR amplicons and sizing standard

simultaneously. Figure 7D shows a fluorescence image of PCR amplicons co-injected with a sizing standard.

Portable instrument: The instrument used to perform analyses with the microdevice is shown in Figure 7E. The instrument contains a 488-nm frequency doubled diode laser, an optical system for detecting four different fluorescence signals, pneumatics for the on-chip PDMS microvalves, electronics for PCR temperature control, and four high voltage power supplies for CE.

(i) Quadruplex mini-Y STR typing: The quadruplex mini-Y STR system included amelogenin and three Y-chromosome STR loci, DYS390, DYS393 and DYS439. The operational details are summarized in the full technical report and in ref. 15. The key findings are summarized below.

Monoplex and multiplex amplifications: We first performed singleplex amplifications on each locus to examine the functionality of the PCR-CE microsystem as well as the amplification performance of these DNA markers. As shown in Figure 8A, each DNA marker demonstrated a similar amplification efficiency and good sensitivity. Following successful amplifications on each locus individually, a multiplex PCR-CE experiment was carried out on this four-locus multiplex system. As shown in Figure 8A, all the peaks (106, 112, 123, 171 and 191 bp) were fully resolved and balanced. Compared with singleplex amplifications, multiplex STR amplifications exhibit lower amplicon yields due to competition between each locus. Therefore, both the initial template copy number (50 copies) and the PCR cycle number (35 cycles) were increased to compensate for this effect.

A limit-of-detection (LOD) analysis for multiplex amplifications of 9948 male standard genomic DNA was performed. Figure 8 presents results from a series of amplifications conducted from 0, 20, 30 and 50 copies of template in the PCR chamber with 35 PCR cycles. Even with only 20 copies of DNA template in the reactor, the multiplex amplification still shows all the expected peaks in the electrophoregram.

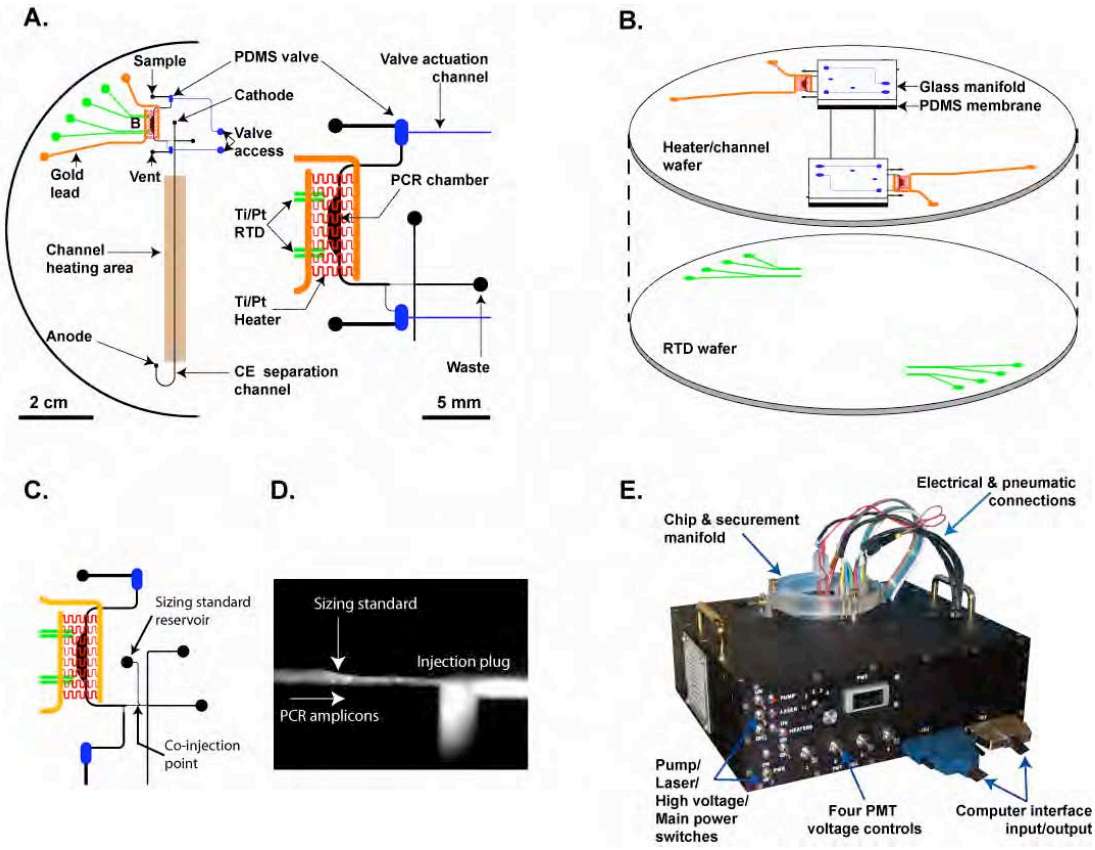

Figure 7. (A) Mask design for the PCR-CE microchip. The glass microchannels are indicated in black, the microfabricated RTD and electrodes are in green, the heater is shown in red, the gold leads of the heater are in gold, and the valves are drawn in blue. (B) Exploded view of the assembly of the PCR-CE microchip, showing the RTDs on the upper surface of the RTD wafer, the glass microchannels etched in the lower surface, and the heaters fabricated on the upper surface of the heater/channel wafer. (C) Design of the PCR-CE chip with a co-injection structure. (D) Fluorescence image of the PCR amplicons and sizing standard co-injection. (E) Photograph of the portable PCR-CE system.

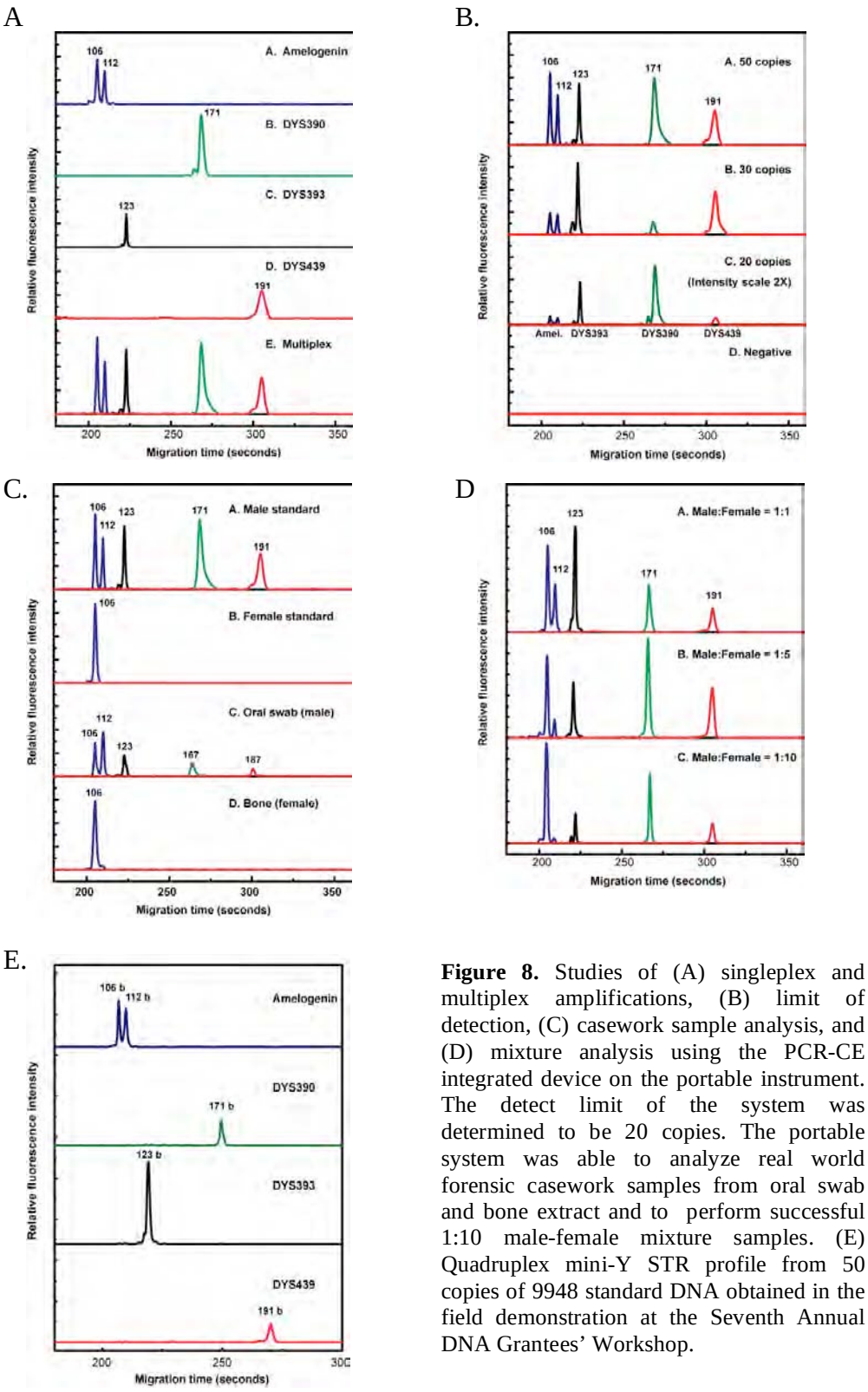

Figure 8. Studies of (A) singleplex and multiplex amplifications, (B) limit of detection, (C) casework sample analysis, and (D) mixture analysis using the PCR-CE integrated device on the portable instrument. The detect limit of the system was determined to be 20 copies. The portable system was able to analyze real world forensic casework samples from oral swab and bone extract and to perform successful 1:10 male-female mixture samples. (E) Quadruplex mini-Y STR profile from 50 copies of 9948 standard DNA obtained in the field demonstration at the Seventh Annual DNA Grantees' Workshop.

Analysis of forensic casework samples: To verify the performance of our portable system on real-world forensic samples, we selected two typical samples, one from an oral swab and the other from human bone, which were previously processed and analyzed by the Palm Beach County Sheriff's Office. Figure 8C (Trace A and B) presents the PCR analyses conducted from male and female standard DNA as controls, showing all the expected peaks with correct gender discrimination. Figure 8C (Trace C) presents an amplification and analysis of the DNA sample extracted from an oral swab. All the amplicons in four loci were successfully obtained, indicating the sample is male DNA. Trace D shows only one peak at 106 bp, corresponding to the successful amplification of female human bone DNA.

Mixture analysis: Mixture analysis was carried out by mixing male and female standard genomic DNA together during the sample preparation. The results of this experiment in Figure 8D show that, as the ratio increased, the 106-bp amplicon from X chromosome became more and more dominant over the 112-bp Y-chromosome product. The peak area ratios are roughly equal to the initial template ratios of Y-to-X chromosomes (1:3, 1:11 and 1:21). The other three Y-chromosome loci (DYS390, DYS393 and DYS439) were still fully amplified and balanced in each case.

Field demonstration: Finally, we carried out a field demonstration of the portable microsystem at the Seventh Annual DNA Grantees' Workshop at Washington, D.C. in 2006. As shown in Figure 8E, a full profile of the quadruplex STR system amplified from 50 copies of 9948 male standard DNA was obtained, demonstrating the portability, robustness and reliability of the system.

(ii) Nine-plex autosomal STR typing: To further extend the application of this portable microsystem, a 9-plex autosomal STR typing system has been constructed using primer sequences employed in PowerPlex® 16. It consists of Amelogenin, and eight STR loci (D3S1358, THO1, D21S11, D5S818, D13S317, D7S820, vWA, and D8S1179) with a size range of 106–258 bp The key findings are summarized in the following:

System characterization: We first investigated on-chip PCR amplification and separation of 9947A and 9948 standard DNA using the 9-plex STR system on the 2nd generation microdevice. Figure 9 shows the successful amplification profiles on 9947A

and 9948 standard DNA obtained from 100 copies of template with 32 PCR cycles in 2.5 hr. using this PCR-CE microdevice. All the alleles of 9947A and 9948 standard DNA were balanced, fully resolved, and correctly sized.

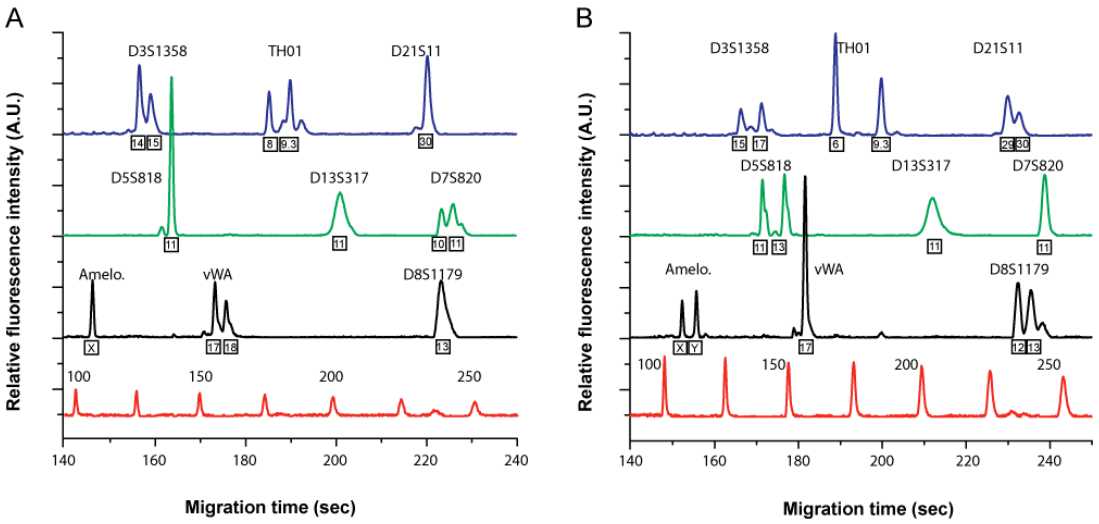

Figure 9. 9-plex STR profiles obtained from 100 copies of (A) 9947A and (B) 9948 DNA on the portable PCR-CE microsystem with 32 PCR cycles. Separation was achieved in 5 % LPA with 6 M urea in a 7-cm microchannel at 250 V/cm.

A limit-of-detection (LOD) analysis for multiplex amplifications of 9947A standard DNA was also performed with serially diluted DNA templates. The portable microsystem was able to produce 100% profiles down to 100 copies of DNA in the PCR reactor.

Our demonstration of successful STR analyses performed on this portable PCR-CE system validates the concept of point-of-analysis DNA typing of forensic casework, of mass disaster samples, or of individuals at a security checkpoint.

Dissemination: We have written or published five papers and made 10 conference presentations on this work. See detailed listing of these activities after the Technical Report.

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DETAILED TECHNICAL REPORT

Review of Literature

The escalating backlog of crime scene evidence in forensic laboratories[1] and the substantially increasing number of items submitted for DNA testing drives the need to automate forensic DNA analysis. However, forensic laboratories face unique challenges in achieving this goal since evidentiary samples are obtained from widely varying substrates, and have different degrees of DNA degradation, amounts of PCR inhibitors, and amounts of DNA. Any automated system used to perform the different steps involved in forensic DNA testing must be flexible and sensitive enough to accommodate the great disparity in DNA quality and quantity. While automated capillary electrophoresis (CE) systems for short tandem repeat (STR) analysis have been in place in forensic laboratories for several years,[2-6] the application of robotic DNA extraction to casework samples[7-9] and automated DNA quantitation systems[10-14] have only appeared recently. It is also desirable to develop a thermocycler and DNA electrophoresis system that can substantially reduce the 3-hour PCR process using current commercial typing systems to improve the turn-around time as well as the number of cases an examiner can complete.

One key issue in forensic laboratories is throughput. The highest throughput CE instrument currently available for forensic analysis is the ABI 3100 Capillary DNA Sequencer that contains only 16 capillaries.[15] In order to complete analysis of a single tray of 96 samples, it still takes many hours for electrophoresis and data capture. Current capillary electrophoresis systems in place, however, are not equipped to sufficiently combine throughput with automation so that data are captured and analyzed in the most efficient, time saving manner.

Another generic problem with capillary array separation systems is the inefficient injection (<1%) of STR fragments when injecting from high salt PCR solutions. This inefficiency is a disadvantage when performing “touch” evidence or low copy number (LCN) or degraded DNA typing. Although increased sensitivity required to successfully type LCN samples can frequently be achieved by use of additional PCR cycles[16-18] or by reduced amplification reaction volume,[19,20] drop-in alleles can be introduced which can potentially interfere with accurate sample profiling. Higher sensitivity fluorescence labeling and more efficient injection are needed to address these difficulties.

The forensic community would benefit from the development of a multi-capillary array electrophoresis system that has integrated sample processing including initially product desalting and concentration before injection, and eventually rapid thermocycling as well as DNA template capture and purification. These capabilities would shorten the time for the thermocycling and electrophoretic process, would increase reliable sensitivity for LCN profiling, and could dramatically reduce the amount of instrument and hands-on time necessary to analyze the STR samples. Moreover, since the DNA sample is placed only once into a well in an integrated system, a fully automated instrument would then carry the sample through the PCR process to electrophoresis and data capture and the combined system could reduce the possibility of sample mix-up and cross contamination.

Many of these needs were critical issues in the Human Genome Project a decade ago and our group conquered them through the development of improved, high-throughput capillary array electrophoretic (CAE) separation systems,[21, 22] through the development of better energy transfer dye labeling systems with 2- to 8-fold sensitivity enhancements[23] and through the utilization of conventional but very useful macro-robotic systems for sample transfer. Until recently, the focus of most new technology development has been on demonstrations of forensic multiplex STR separations in single channel devices.[24, 25] In 2006, our group established a milestone in high-throughput, high-quality forensic STR analysis using a 96-lane microfabricated capillary array electrophoresis (μCAE) system. The successful demonstration of the microdevice and collaboration with the Virginia Department of Forensic Science as well as the Palm Beach County Sheriff's Office led the eventual technology transfer and validation of a prototype μCAE instrument at VDFS.[26] This achievement is a major step in migrating towards microchip utilization for forensic DNA typing and sets the stage for the goal of incorporating the immediate upstream PCR processes to achieve a fully integrated STR analysis system.

Over the past three years the state-of-the-art in integrated sample processing has dramatically advanced in our lab. The keys to this success were (i) the development of pneumatic PDMS valve structures for reliable fluidic containment, valving and pumping,[27] (ii) the development of precisely microfabricated and reliable temperature sensors and integrated heater systems,[28] and (iii) the development of integrated

oligonucleotide-gel capture matrices for PCR product purification.[29] We have presented an integrated microfluidic system for Sanger sequencing suggesting that the entire process can be performed with at least a 10-fold volume reduction.[30] If these same technologies can be applied to forensic identification, then a scaleable paradigm for integrated forensic DNA analysis will be in hand that can empower both small and large labs with the most modern technical capabilities.

These technologies are sufficiently robust that they can be combined into complex integrated circuits to perform a wide variety of genetic analysis tasks. For example, Blazej *et al.*[31] performed integrated thermal cycling in a 200 nL reactor coupled to integrated oligo-gel capture of the sequencing products and efficient (10%) injection producing over 500 base reads from only 1 femtomole of template. More recent work perfected an in-line capture and injection strategy that provides quantitative capture and injection of all of the thermally cycled products enabling sequencing from only 100 attomoles of template.[32] Sequencing and genetic analysis microdevices have also been extended to multiple reactors and separation channels. Liu *et al.* developed a four separation channel structure where each channel is coupled to an integrated PCR reactor.[33] This device performed parallel multiplex PCR amplification, injection and separation of the ds-DNA products in under 30 min with 10-copy sensitivity. This four channel device was also modified to perform multiplex one-step RT (reverse transcriptase) PCR analysis of RNA targets.[34] This device demonstrated sensitive (10 copy) RNA analysis and multichannel multiplex RNA transcript analysis. Only the IR thermal cycler system by Landers has been similarly extended to perform complex sample-to-read analysis of infectious disease.[35] It is notable that they accomplished this by using the PDMS valve structures developed in our group for fluidic control (following training at Berkeley). Clearly the separation, sample processing and target amplification technologies that have now been developed provide a remarkable robust platform that is ready to be transferred to address the needs of forensic analysis.

Research Methods, Findings and Conclusions

1. *Evaluation of μCAE performance with commercial STR typing systems*

A 96-channel microfabricated capillary array electrophoresis (μCAE) system as shown in Figure 1 was evaluated for forensic short tandem repeat (STR) typing using PowerPlex 16® and AmpFℓSTR® Profiler Plus® multiplex PCR systems in collaboration with VDFS and PBSO. Performance evaluated included analysis speed, fragment resolution, profile concordance, mixture allele discrimination, sensitivity and real-world forensic sample typing capability. Results of this work and the technical details were disseminated in the *Journal of Forensic Sciences.*[36] A copy of the paper is included in the Appendix. A summary of the key findings and technical details of this work is provided in the following.

All microfabrication performed followed procedures detailed in Yeung *et al.*[36] Briefly, the microdevices were fabricated on 150-mm diameter borofloat glass wafers (Schott, Yonkers, NY) using standard photolithographic method. After photolithography, all features were isotropically etched to a depth of 25 μm with hydrofluoric acid. The post-etch width is 85 μm for the arm from the sample to the separation channel, 300 μm from the waste to the separation channel, and 200 μm for the separation channel connecting the cathode and the central anode. The fluidic wells were diamond-drilled into the etched wafers using a CNC mill. The wafer was then cleaned and thermally bonded to a blank wafer to create a closed channel sandwich structure.

All DNA and PCR samples were prepared by PBSO using standard procedures following manufacturer protocols. Aliquots of the amplified samples were shipped to Berkeley and VDFS for independent analyses on the μCAE system and an ABI 310, respectively. Analysis of DNA profiles generated using the μCAE system was performed using the Amersham MegaBACE Fragment Profiler (GE Healthcare, Piscataway, NJ).

Speed, Resolution & Concordance: The high-throughput μCAE system produced high-speed (< 30 min) parallel sample separations with single-base resolution based on the 9.3/10 THO1 alleles in the PowerPlex 16 allelic ladder. Forty-eight single-source samples (28 Hispanic, 3 Asian and 17 African American) previously analyzed by PBSO were accurately typed, as confirmed on an ABI Prism 310 and/or the Hitachi FMBIO II.

Mixture study: STR samples consisting of male and female DNA at the ratios of 10:0, 9:1, 3:1, 3:2, 2:3, 1:3, 1:9 and 0:10 were analyzed. The 3:1 and 1:3 samples are the lowest (highest) ratios in which all minor components were detected and reliably typed.

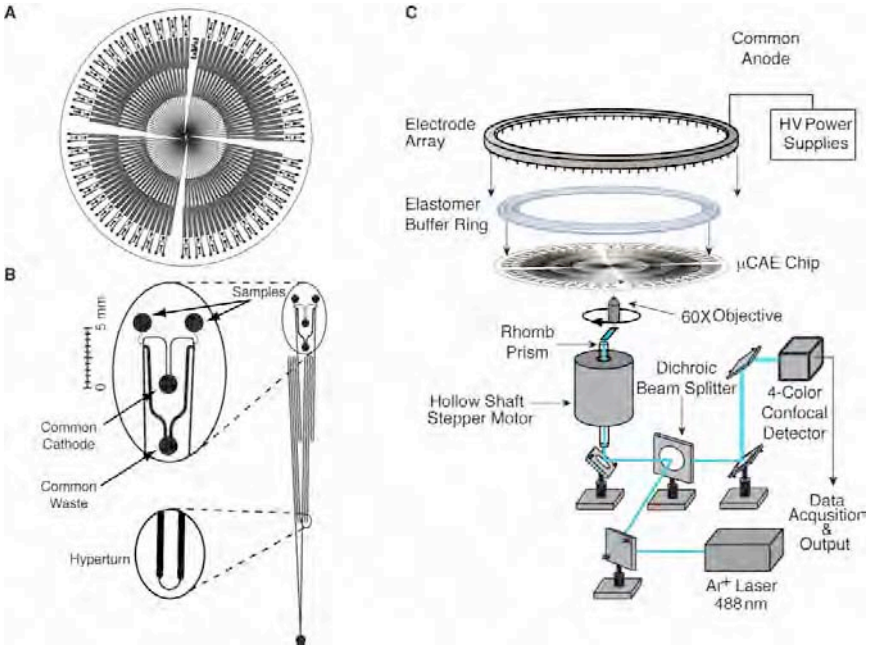

Figure 1. (A) Layout of the 96-lane μCAE chip. The 96 adjacent microchannels are grouped into 48 doublets on a 150-mm diameter glass wafer. (B) Each doublet contains two cross-injector structures with two sample wells that share a common cathode and waste wells. During electrophoresis, the sample plug is formed at the intersection of the sample and waste arms and migrates down through the hyperturns towards the central anode. (C) The rotary confocal fluorescence scanning system consists of a 488-nm argon ion laser beam which is passed through a dichroic beam splitter and directed 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 an objective on the microchannels. The emitted fluorescence travels back along the same path into a 4-color confocal detector. A polydemethlysiloxane (PDMS) elastomer ring is placed on top of the μCAE chip to create a continuous buffer reservoir for the cathode and waste wells. An electrode ring is used to supply voltage to sample wells.

Sensitivity study: The sensitivity of the μCAE system was assessed using PowerPlex 16 and Profiler Plus samples amplified with serially diluted DNA templates (22, 11, 5.5, 2.75, 1.38, 0.69, 0.34, 0.17, 0.08, 0.043, 0.021, 0.011, and 0.0054 ng). The instrument produced full profiles from sample DNA down to 0.17 ng, yielding 100% of the expected profiles for both tying kits; a threshold similar to that found for the ABI 310.

Forensic DNA sample typing: Seventeen non-probative samples were correctly typed from case evidence previously processed and analyzed by PBSO using both the PowerPlex 16 and Profiler Plus systems. The DNA extracts were collected from a variety of common stain sources encountered in forensic analysis, including semen, saliva, single, and mixed blood stains from sexual assault, paternity, burglary, armed robbery as well as homicide cases. The DNA data obtained using the μCAE system for the less

complicated paternity and single-source bloodstain case samples produced full profiles that matched with the previously reported case results. For the sexual assault DNA samples, both μCAE and ABI 310 profile results for the sperm-fraction obtained from the vaginal-swab semen agreed with the original report analysis - the DNA profile did not match the suspect's DNA profile. However, the μCAE system was able to discern additional alleles in the nonsperm fraction of the semen stain, consistent with the semen contributor that were not callable previously using the FMBIO II. For the bloodstain mixture collected from a knife blade and another from a sandal in a homicide, the μCAE profile results were identical to the original report as well as to the ABI 310 analyses, even at loci with imbalanced peak heights indicating major and minor contributors.

This work successfully demonstrated that current standard commercial forensic typing kits can be effectively applied to our μCAE system with no modification - a significant step and knowledge gain towards the ultimate goal of developing of a completely integrated forensic STR typing microsystem.

2. Development and validation of a prototype μCAE forensic scanner for VDFS

A prototype μCAE chip scanner system from GE Healthcare (formerly Amersham Bioscience) has been successfully installed in VDFS and validated by VDFS forensic scientists in July 2007. The work on validation has been submitted to the *Journal of Forensic Sciences* and included in the Appendix.[37] The system consists of a four-color microchannel plate (MCP) scanner (Fig. 2A), a gel/sample loader and a chip cleaning station (Fig. 2B and 2C), and was first delivered to the Mathies Lab at Berkeley in April 2005. There were three major stages in achieving this goal.

In the first stage, we performed initial testing on the scanning system for STR typing operation, troubleshot and identified the needs of additional software for MCP plate focusing on the stage as well as electrical current monitoring functions. We also tested the gel/sample loader and chip cleaning station for their ability to perform the appropriate functions as expected. In December 2005, forensic scientists Susan Greenspoon and Amy McGuckian visited Berkeley for their first training on the system, provided feedbacks and suggestions on areas and functions to be improved and

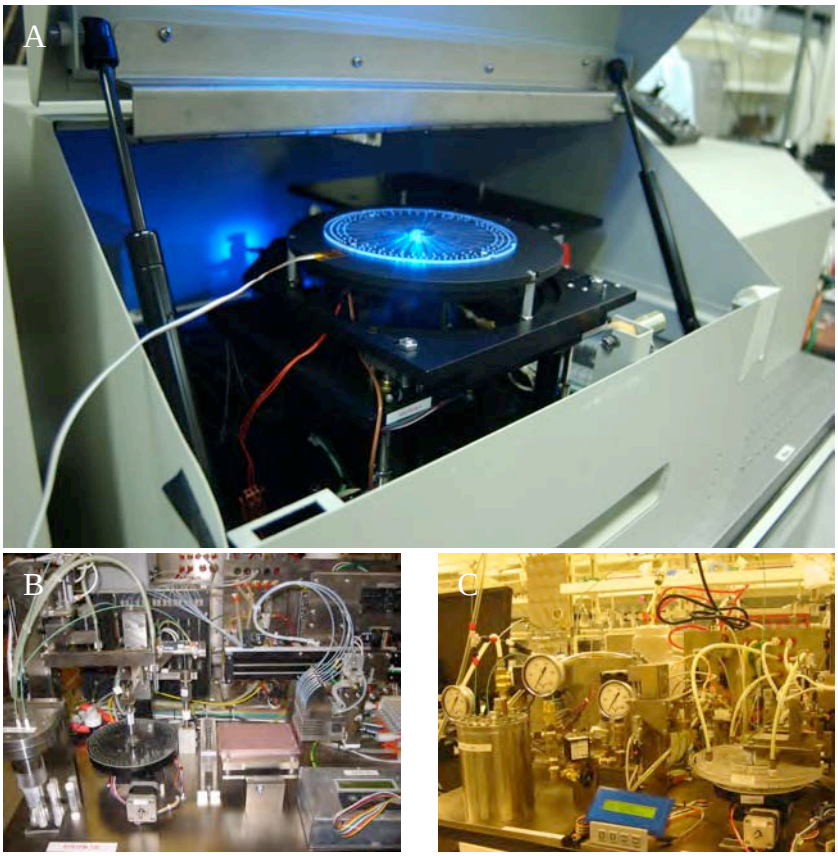

Figure 2. Pictures of the MCP system at VDFS consisting of (A) a four-color confocal scanner, (B) a gels/sample loader and (C) a microchip washing station.

incorporated. These included further modifications on the gel/sample loader for better gel use monitoring and on the data acquisition/processing software to better streamline with the DNA profiling analysis software —MegaBACE Fragment Profiler.

The second stage marked the transfer of the MCP system outside of a research setting laboratory to a forensic one. In Feb 2006, Susan Greenspoon from VDFS visited the Berkeley lab again for a final hands-on training and demonstrated that she could perform successful STR typing repeatedly without the intervention of Berkeley scientists. The MCP system was shipped and installed at VDFS in April 2006 followed with fine tuning and minor adjustments of the operation procedures for a forensic laboratory setting.

The final stage marked the mastery of the MCP system by VDFS forensic scientists and its validation for forensic STR typing. Standard validation assays performed include resolution measurements, concordance, sensitivity and mixture studies as well as non-probative and Y-STR sample typing. We have also examined the success rate of the 96-lane system and investigated alternative microchannel coating methods for

more user-friendly operation. STR data produced on both the μCAE device and the ABI 310 were compared. The data produced from the μCAE device were sent to PBSO for comparison with previous DNA profiling results for the cases as well. Twenty one μCAE STR trace sets were also sent out to Cybergenetics for analyses on True Allele® System which generated identical results as those obtained on the MegaBACE Fragment Profile (GE Healthcare) genotyping software.[37]

All PCR samples were amplified using the PowerPlex® 16 or the PowerPlex Y system following the manufacturer's recommendations [38, 39] on a GeneAmp System 9600 cycler (except as noted otherwise). The STR amplicons were electrophoresed on both the ABI Prism® 310 Genetic Analyzer and the μCAE device. Preparation of samples for electrophoresis on the ABI 310 was as follows: 1 μL of each PCR product was added to a loading cocktail containing 24 μL Hi-Di™ Formamide (ABI) and 1 μL Internal Lane Standard 600 (ILS600) (Promega Corp.). One allelic ladder sample was included for approximately every 15 samples. The ladder was prepared in the same manner as PCR products with 1.0 μL PowerPlex® 16 or PowerPlex® Y Allelic Ladder Mix added to the loading cocktail. Samples were denatured for 3 minutes at 95°C and snap-cooled on ice prior to loading.

Sample preparation for μCAE analysis was as follows: 1 μl of each PCR product was mixed with 3 μL Hi-Di™ Formamide, 3 μL sterile Type 1 H_2O, and either 1 or 0.75 μL ILS600 following the procedure used by Yeung *et al.*[36] The ladder was prepared by adding 2.0 μL PowerPlex® 16 Allelic Ladder Mix or PowerPlex® Y Allelic Ladder Mix (for PowerPlex® Y amplified samples) to a cocktail containing 2.5 μL Hi-Di™ Formamide, 2.5 μL sterile Type 1 H_2O, and either 1 or 0.75 μL ILS600. Samples were denatured for 3 minutes at 95 °C and snap-cooled on ice prior to loading.

For the ABI 310 analysis, the operation of the instrument followed the manufacturer's directions for use with the STR typing kits.[38, 39] The raw data were collected with ABI Data Collection Software and analyzed using GeneScan and Genotyper software, versions 3.1 and 2.5, respectively. Allele calls were performed using the PowerTyper™ 16 or PowerTyper™ Y Macros (Promega Corp.).

Operation of the μCAE device followed the procedures outlined by Yeung *et al.*[36] For μCAE chips coated using the modified Hjerten procedure,[40] a fresh coating was

applied every two weeks (S. Yeung, personal communication). For μCAE chips coated using the polyDuramide (pDuramide) dynamic coating polymer,[41] the procedure for coating was as follows: μCAE chips were first flushed with deionized sterile water (dH2O) from the central anode to distribute fluid to all capillaries, then the microchannels were filled with 1 M HCl for 15 minutes followed with a dH2O flush. The microchannels were then filled with pDuramide coating solution and incubated for 15 minutes, followed with a dH2O flushed with then dried for use. A fresh coating was applied every 5 days.

The following highlights the findings and technical details associated with each study.

Precision, Resolution, Success Rate & Concordance: Precision results obtained by separating PowerPlex 16 allelic ladder samples on the μCAE instrument were compared to those obtained on ABI 310 at VDFS as well as on commercial multi-capillary instruments previously. The single capillary ABI 310 at VDFS displayed better precision than the μCAE as well as data previously obtained at Berkeley. However, the precision of μCAE is essentially equivalent to commercial CAE systems (ABI 3100, 3700 and MegaBACE 100) as shown on Table 1.

Resolution measurements were obtained based on the TH01, CSF1PO, TPOX & Amelogenin loci in PowerPlex 16 allelic ladder using methods detailed in Buel *et al.*[42] Briefly, resolution results as shown in Table 2 obtained using μCAE were similar to those reported by various sources for ABI 310 as well as those measured for μCAE by Yeung *et al*[36] using the TH01 locus. Resolution measurements for both Hjerten and pDuramide coating methods were nearly identical and were similar to those produced for the μCAE at Berkeley. Peak morphology and separation between the 9.3 and 10 alleles of the TH01 allelic ladder were virtually identical between the two coating procedures and similar to that produced by ABI 310. In addition, the pDuramide coated μCAE microchips (8 runs) demonstrated a greater number of open capillaries (~ 20% more) than the modified Hjerten coated microchips (7 runs).

Table 1. Sizing Precision

Instruments	Sizing precision (S.D.) Within-run	Between-run	± 3 S.D.
ABI 310 (VDFS)	0.03-0.06 bp	0.03-0.06 bp	± 0.18 bp
μCAE (VDFS)	0.02-0.23 bp	0.08-0.14 bp	± 0.42 bp
μCAE (Mathies' lab data)	---	0.11-0.31 bp	± 0.93 bp
ABI 377[5]	0.01-0.09 bp	---	---
ABI 377[43]	0.03-0.10 bp	---	---
ABI 310[2]	---	0.02-0.12 bp	± 0.36 bp
ABI 310[5]	---	0.04-0.12 bp	---
ABI 310[44]	0.10 bp	0.20 bp	---
ABI 310[45]	0.01-0.13 bp	≤0.16 bp	---
ABI 3100[43]	---	0.03-0.17 bp	---
ABI 3700[43]	---	0.02-0.21 bp	---
FMBIO II[46]	---	---	± 0.40-0.80 bp
MegaBACE 1000[43]	---	0.04-0.17 bp	---

Table 2. Measurements of Resolution

Instruments	Rb	RSL	Vv
ABI 310 (VDFS)	1.15-1.72	0.67-0.99	0.64
μCAE (Mathies' lab data)	1.30-1.61	0.74-1.04	0.73
μCAE (VDFS) Hjerten coating	1.35-1.53	0.78-0.91	0.80
μCAE (VDFS) pDuramide coating	1.31-1.54	0.78-0.904	0.74
ABI 310[42]	1.04-1.64	0.61-0.96	0.51
ABI 310[45]	1.24-1.31	---	0.43-0.49
ABI 310[5]	---	---	~0.30
ABI 310[3]	1.13-1.49	---	---
μCAE[36]	1.3	0.76	---

For the concordance study, 47 single-source DNA samples were extracted and purified from buccal swabs and dried blood cards by VDFS using the DNA IQ™ System (Promega Corp., Madison, WI) according to the manufacturer's protocol with minor modifications for buccal swab and blood stains samples as described[47] and outlined in the VDFS procedure manual[48] or using an organic extraction, followed by Micron YM-100 clean-up as described in the manual. Full concordance was obtained for all 47 DNA profiles as confirmed using the ABI 310 and by the VDFS staff DNA index.

Sensitivity & mixture studies: The DNA samples were purified from either buccal swabs or tissue samples as defined[47] using the robotic DNA IQ™ extraction procedure or an organic extraction method as described above. The sensitivity study was performed on microchip coated with the Hjerten and pDuramide dynamic coating procedure using a sensitivity series provided by NIST (10, 5, 2.5, 1.25, 0.63, 0.31 and 0.15 ng) and VDFS (2, 1, 0.5, 0.25, 0.125, 0.062, 0.031 & 0.015 ng), respectively. As demonstrated in Table 3 and Figure 3, the results showed that the sensitivity of the μCAE device was not

affected by the coating and comparable to that reported for commercial CE instruments[17,49] and to that reported by Yeung *et al.*[36]

In mixture study, PowerPlex 16 samples amplified using mixed DNA samples prepared by VDFS at the ratios of 1:0, 9:1, 3:1, 3:2, 2:3, 1:3, 1:9 & 0:1 were analyzed using microchip coated with pDuramide. The majority of minor contributor alleles were detected at the 3:1 and 1:3 ratio samples. While all minor components were detected in these samples previously in Yeung *et al.*[36], alleles below threshold were observed for allele 12 at D3S for the 3:1 samples, as for allele 9 at TH01 and allele 11 at Penta E for the 1:3 sample. These differences were possibly due to the differences in methods used in estimating DNA concentration by VDFS and by NIST in Yeung *et al*.[36] For both the 9:1 and 1:9 samples, minor contributor alleles were above the peak threshold at many loci, consistent with other reports.[36, 46, 49, 50]

Non-probative & Y-STR analyses: PowerPlex 16 samples of 19 non-probative DNA extracts from five cases (two sexual assaults, a hit-and-run, aggravated battery, and aggravated robbery/aggravated battery) were prepared. All DNA samples were quantified at PBSO with Quantifiler™ Human DNA Quantification Kit (ABI), on a Biomek® 2000 Laboratory Automation Workstation (Beckman Coulter, Inc., Fullerton, CA) for qPCR reaction set up. Fluorescent signals were detected using the ABI 7000 following the manufacturer's recommendations.[51] Although DNA profiling was originally done using various PCR-based human identification kits, 100% concordance was obtained for μCAE data compared to the original case reports (C. Crouse, personal observations). As shown in Figure 4, the minor contributor alleles from the sperm DNA which carried over into the non-sperm fraction are clearly visible and the major profile is consistent with the victim.

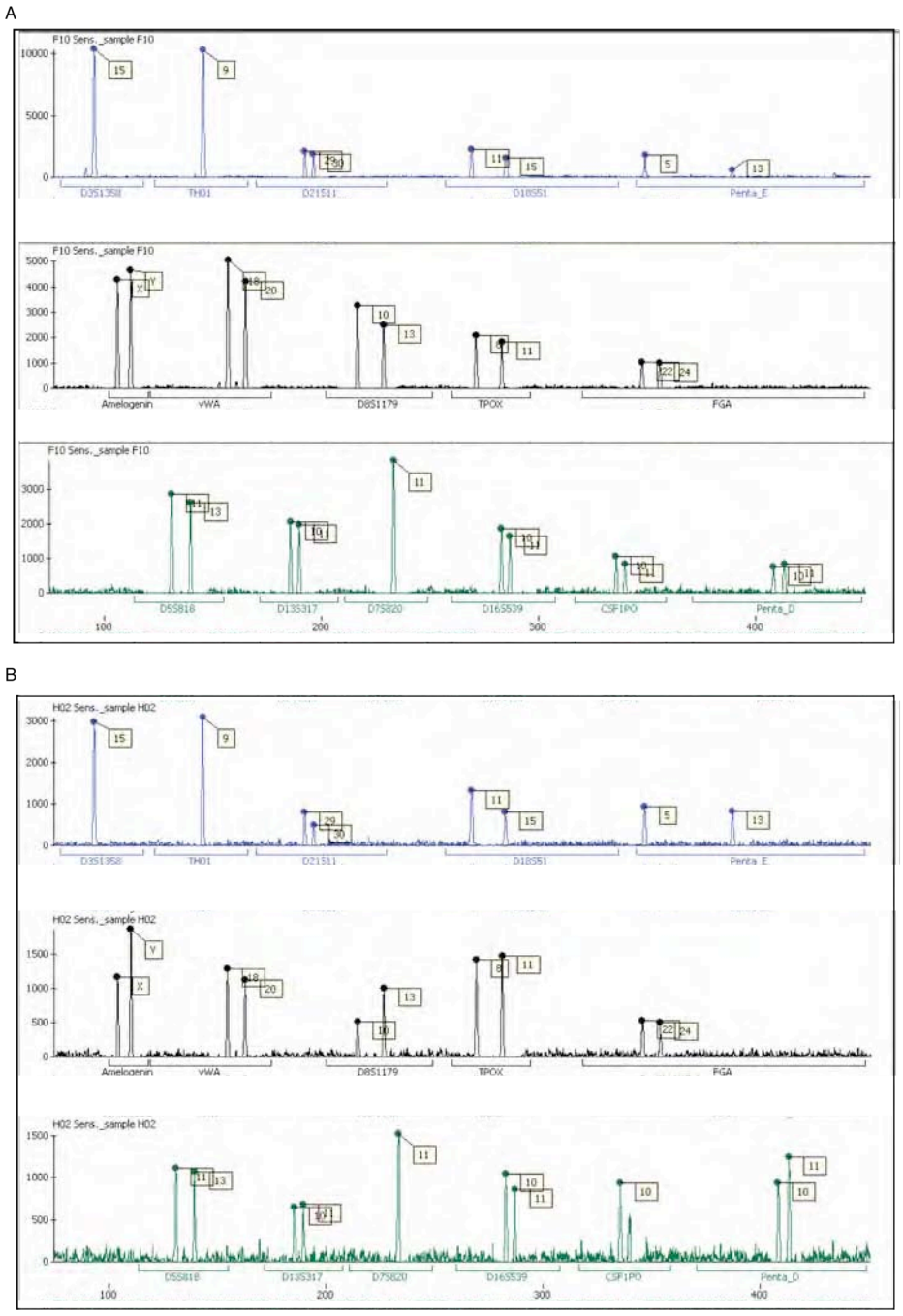

Figure 3. PowerPlex 16 profiles of sensitivity study samples using a single-source male DNA sample amplified with (A) 2 ng of input DNA and (B) 125 pg of input DNA. (Top: Fluorescein channel, Middle: TMR channel, Bottom: JOE channel.)

Table 3. Sensitivity Data.

VDFS DNA sample – pDuramide coating

	FGA	TPOX	D8	vWA	Amel	P.E.	D18	D21	TH01	D3	P.D.	CSF	D16	D7	D13	D5
In house sample																
2 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
1 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
0.5 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
0.25 ng	22,24	8,11	10,13	18,20	XY	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
0.125 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10	10,11	11	10,11	11,13
0.062 ng	-	-	-	-	X,Y	-	11	-	9	15	-	-	-	-	-	
0.031 ng	-	8,11	-	18	X,Y	5	15	-	9	15	-	-	-	-	-	-
0.015 ng	-	-	-	-	Y	-	-	-	9	15	-	-	-	-	-	-

NIST sample – modified Hjerten coating

	FGA	TPOX	D8	vWA	Amel	P.E.	D18	D21	TH01	D3	P.D.	CSF	D16	D7	D13	D5
NIST sample																
10 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
5 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
2.5 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
1.25 ng*	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
0.62 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
0.31 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
0.15 ng	19	8	14	17,18	X,Y	5	14,16	31.2,33.2	7	16	14	12	9	11	-	-

*Data for sample obtained from a μCAE device run performed on a different day.

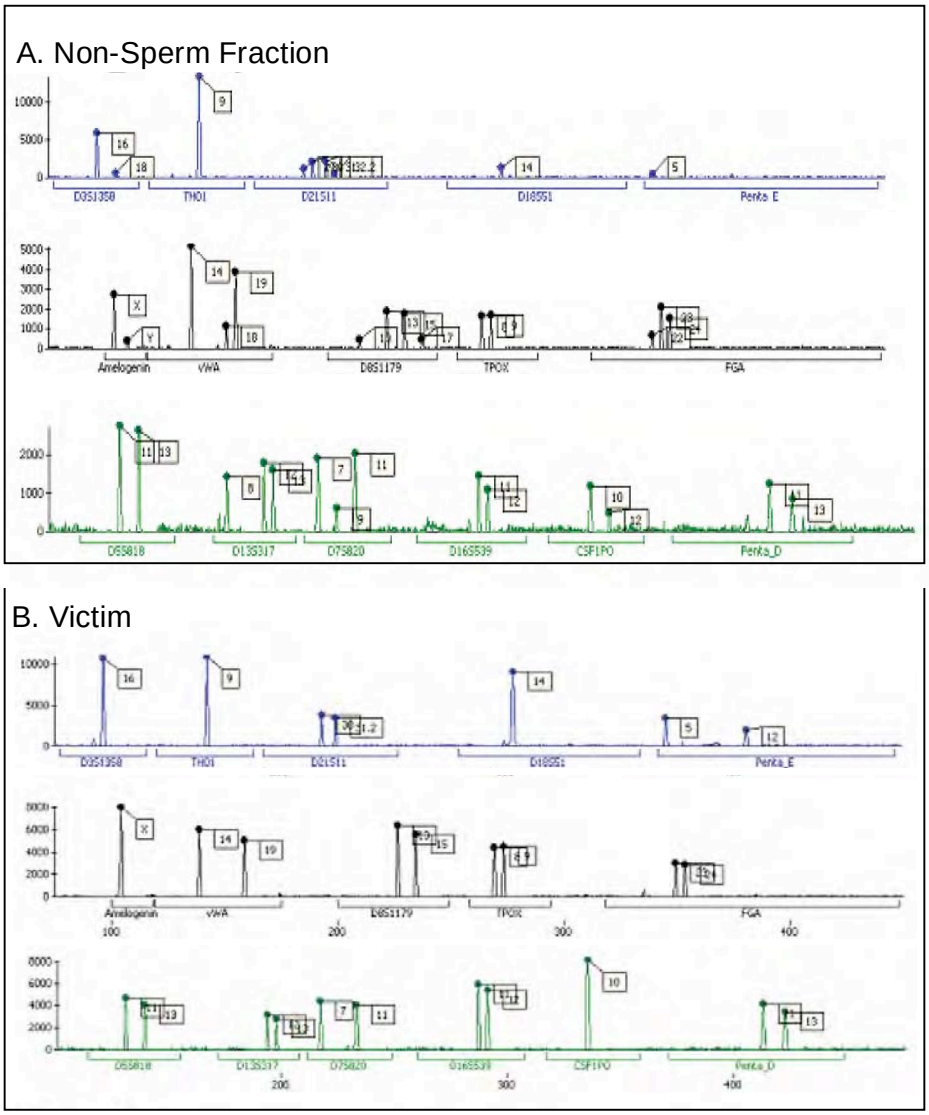

Figure 4. PowerPlex 16 profiles of (A) Non-sperm fraction mixed sample and (B) victim DNA sample obtained using the μCAE device. Top: Fluorescein channel, Middle: TMR channel, Bottom: JOE channel.

In a Y-STR typing study, 12 PowerPlex Y samples were prepared from DNA extracts of mock sexual assault stain samples created by placing amounts of 1,000 (1K), 10,000 (10K) or 50,000 (50K) spermatozoa on a half-epithelial swab (buccal or vaginal) provided by Promega Corporation. DNA samples were extracted either with the semi-automated Differex™ method according to the vendor's protocol[52] or as described,[47] and were quantified with the AluQuant® Human DNA Quantitation System (Promega Corp.).

A Biomek® 2000 Laboratory Automation Workstation (Beckman Coulter, Inc., Fullerton, CA) was used to set up the enzymatic reaction and the Luminoskan luminometer (Thermoelectron, West Palm Beach, FL) to detect the signal produced. DNA profile results were concordant between the two DNA separation instruments with some of the lower-signal samples (peak heights close to the 100 rfu threshold on the ABI 310 – or S/N < 3:1 for the μCAE data) not labeled. Note that no formal threshold for forensic STR typing has been established for the prototype μCAE instrument yet. Nonetheless, majority of the mock sexual assault samples provided STR profiles of similar high quality to those produced by the ABI 310.

The successful transfer the prototype μCAE system to a forensic laboratory setting and its application to actual forensic STR typing samples without significant modifications further demonstrate its functionality and attests to ease of use of the final instrument and protocol. This milestone also establishes a significant step towards the development of a completely integrated STR analysis microdevice. A review paper on this topic and the vision of integrated microchip technology for forensics will be published Promega's *Profiles in DNA* to inform the forensic community (Greenspoon, S.A., Yeung, S.H.I., Ban, J.D. and Mathies, R.A. Microchip capillary electrophoresis: A step towards DNA integrated forensic analysis system, Profiles in DNA, Promega Corp.).

3. Development of energy-transfer (ET) cassette labeled multiplex typing systems

A 16-plex STR typing system has been developed[53] with improved energy-transfer fluorescent dye cassette labels using primer sequences in the PowerPlex 16 kit. This kit was chosen because of its readily available sequence information in the public domain.[49] To maintain similar dye-induced mobility shifts, FAM labeled loci were replaced with FAM-FAM cassette, JOE with a FAM-R6G cassettes, and TMR with FAM-TAMRA cassettes. Each ET primer was subject to systematic monoplex PCR and μCAE evaluations to ensure it was yielding the expected increase fluorescent signals before it was included in the multiplex. Before multiplex construction, we also looked at the effective annealing temperatures of three cassette labeled primers to show that ET-primers behaved like the single-dye primers. We then compared the performance our ET 16-plex with PowerPlex 16 to evaluate its ability to produce higher STR amplicon

fluorescence signals from low-level DNA, to amplify DNA templates at reduced PCR cycle counts, and to perform typing on challenging DNA samples extracted from variety of forensic evidence. All μCAE operation and analysis procedure follow Yeung *et al.*[36] except for microchannel coating procedure which follows a pDuramide coating procedure described in *Development and validation of a prototype μCAE forensic scanner for VDFS* session above. The findings and technical details are summarized below for each study.

Annealing temperature study: The effective annealing temperature of three cassette labeled primers were investigated and determined to be 60 ^{o}C. The study characterized five annealing temperatures (58, 59, 60, 61 and 62 ^{o}C) of energy-transfer (ET) cassette-labeled primers for three loci, (TH01, Penta D and vWA); each primer was coupled to a different ET-cassette label and compared in parallel to the corresponding single-dye labeled primers. The FAM labeled TH01 primer was compared with the FAM-FAM label; the JOE labeled Penta D with FAM-R6G; and the TAMRA labeled vWA with FAM-TAMRA. All PCR reactions (25-μL) were prepared with 1 ng of 9947A DNA and amplified with 0.4 μM fluorescent primers for thirty-two cycles following the manufacturer recommended conditions for PowerPlex 16. Equal fractions of the ET- and single-dye labeled PCR products amplified at the same annealing temperature for each locus were mixed with the ILS 600 and injected for μCAE analyses using the same conditions detailed in Yeung *et al*.[36] Each mixed sample was loaded into two to four microchannels and the averaged signal-to-noise (S/N) ratio for each allele was investigated as a function of the annealing temperature at each locus. The results of this study show that the ET-primers and single-dye primers follow similar annealing trends. It is observed that 60 ^{o}C is the most effective annealing temperature for all three ET-cassette labels.

Increased fluorescent signals: Equimolar ET-cassette labeled STR primers were electrophoretically separated together with the corresponding single fluorescent-dye labeled primers. ET-cassette labeled primers yield 1.5–8X higher fluorescence intensities as shown in Figure 5A. To examine the effects of thermal cycling on ET-labeled amplicon signals, amplified PCR products were produced with both types of dye labeled primers on three STR loci under identical conditions. The amplified ET labeled PCR

products displayed the expected increase in fluorescence intensities as presented in Figure 5B. Mobility shifts (10.1 bases for FAM-FAM, 9.9 bases for FAM-R6G, and 8.9 bases for FAM-TMR) were observed for the ET-cassette labeled products as a result of the extra fluorescent dye and the sugar phosphate backbone structure in the ET cassette. These results confirm that higher fluorescence-signal PCR products can be achieved using ET-cassette labeled primers under the same PCR conditions as conventional single fluorescent-dye primers.

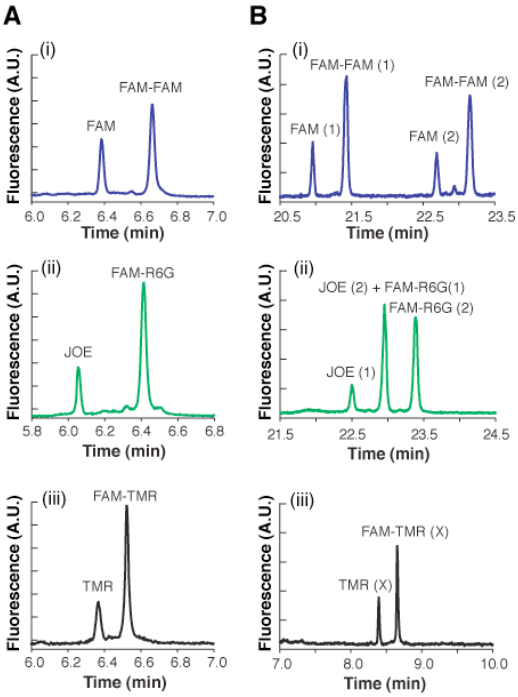

Figure 5. (A) Electropherograms of equimolar ET-cassette labeled primers and their corresponding single-dye labels. (i) FAM-FAM primer gives ~1.5X increase in intensity vs. FAM primer, (ii) FAM-R6G gives ~2.5X vs. JOE and (iii) FAM-TMR gives ~ 3X vs. TMR. (B) Electropherograms of equal fraction of monoplex PCR reactions amplified with ET cassette labeled primers and single-dye labeled primers.

The ET 16-plex typing system was constructed first using the same primer concentrations in PowerPlex 16 (Promega, personal communication) followed with subsequent adjustments of primer concentrations by typing of a single-source DNA sample, A-18 obtained from Promega. The final ET 16-plex primer concentrations used were either the same or lower than those in PowerPlex 16 with equal forward and reverse primer concentrations (except for vWA which is 2X the concentration of the commercial system for ease of balancing). A 10X single-dye primer mix was also prepared using the same primer concentrations for in-house control. All DNA samples were previously purified by Promega or PBSO using standard DNA extraction protocols. All assays were performed with 0.5 ng of input DNA in 12.5-μL reactions for 31 cycles unless noted

otherwise. For multiplex construction, the PCR reaction (0.5 μL) was mixed with 1 μL of ILS 600 and brought up to 18 μL in 50% formamide prepared in deionized water. For all other studies, the PCR (1 μL) reaction was mixed with 1 μL of ILS 600 and brought up to 8 μL with 50% formamide. μCAE analyses followed the previous description in Yeung *et al.*[36]

Representative A-18 profiles generated with both ET 16-plex and the PowerPlex 16 kit are shown in Figure 6. ET-cassette labeled STR alleles yielded 1.6–9X higher fluorescence intensities than single-dye labeled alleles amplified with PowerPlex 16. The FAM dye peak observed on the ET 16-plex samples is a contaminant that could be removed by more rigorous ET-cassette- primer purification. Figure 7 illustrates the increased signal-to-noise (S/N) ratios of 2–6.5X for FAM-FAM loci, 1.6–6.6X for FAM-R6G loci and 2–8.7X for FAM-TMR loci compared to the corresponding single-dye labeled fragments. The increased signal intensity at an STR locus is the averaged ratio of the ET-allele S/N to the single-dye labeled allele based on two data sets (four alleles for heterozygotes and two alleles for homozygotes) from identical PCR reactions analyzed within the same μCAE run.

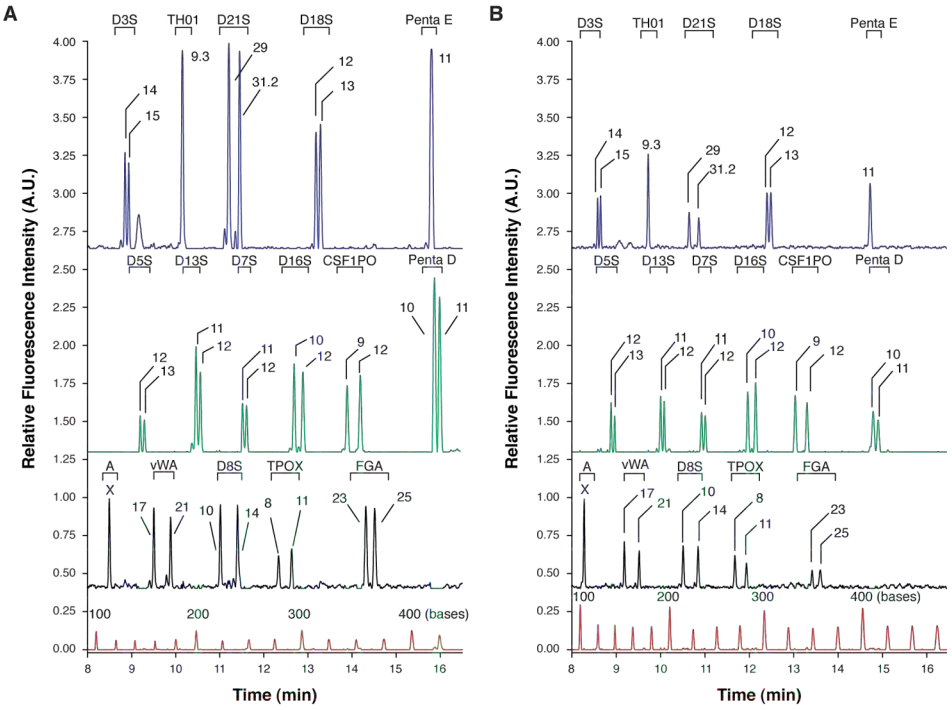

Figure 6. Profiles of 0.5 ng of A-18 DNA amplified with (A) ET 16-plex and (B) PowerPlex 16. The minor dye impurity observed at ~ 9 minutes in some of the ET 16-plex traces does not interfere with any of the calls and is easily removed by additional purification.

Sensitivity study: The sensitivity of the ET 16-plex STR typing system was assessed and compared to PowerPlex 16 using samples amplified from a dilution series of A-18 DNA. Figure 8 plots the percent allele detection from 0.25 to 0.0039 ng of DNA template. All 29 expected STR alleles (defined as S/N ≥ 3) are achieved with the 62.5-pg samples amplified with the ET 16-plex. At the same DNA input, only 79% and 72% of the profile was successfully typed from the PowerPlex 16 and the in-house SD 16-plex samples, respectively. With only 31.3 pg of DNA we could still detect ~97% of the profile using the ET 16-plex. This increase in sensitivity over the single fluorescent-dye labeled multiplex systems demonstrates the capability of ET primers to achieve higher signals or the same signals with less template DNA. This achievement should advance low-copy number (LCN) typing by eliminating the need for increased thermal cycles which frequently results in non-specific amplification. At the same time, this increased sensitivity should improve typing of poor quality DNA samples by making minor or imbalanced alleles more readily detectable at the LCN threshold.

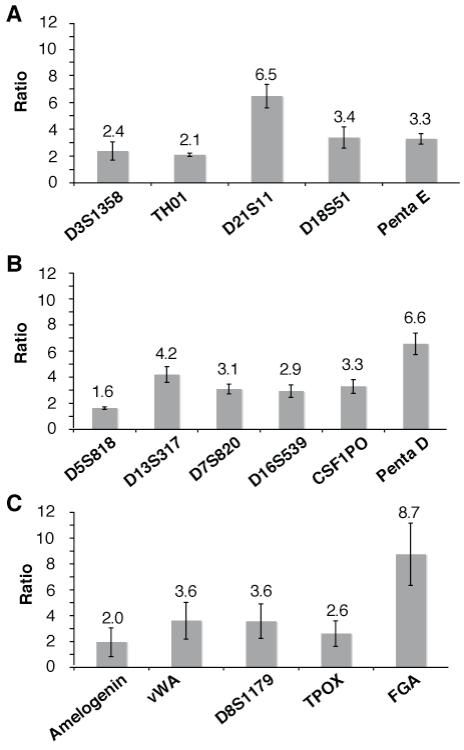

Figure 7. Average fluorescence intensity ratios of ET labels versus single-dye labels at each STR locus. (A) 2–6.5X for FAM-FAM labeled loci, (B) 1.6–6.6X for FAM-R6G loci and (C) 2–8.7X for FAM-TMR loci.

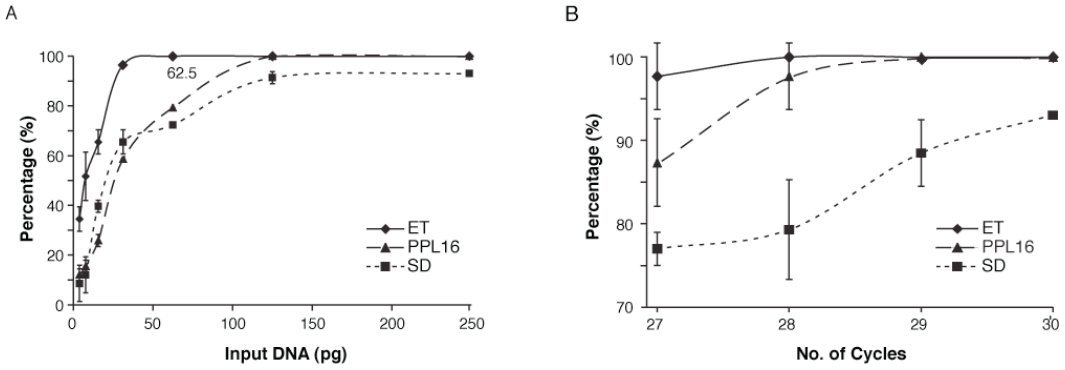

Figure 8. Percentage of full STR profiles obtained for DNA samples amplified with ET 16-plex, single-dye 16-plex and PowerPlex 16 as a function of (A) amount of input DNA and (B) the number of PCR cycles.

Reduced PCR Cycle Study: The traditional approach for performing STR typing of poor-quality DN is to increase the number of PCR cycles, but this can evoke false amplification or allele drop-in.[16-18, 55] With the increased sensitivity of ET labels, we demonstrated the possibility of reducing the total number of PCR cycles to speed analyses and reduce non-specific background. We performed experiments to type 0.5 ng of DNA template at 30, 29, 28 and 27 cycles. Figure 8B illustrates that full profiles were obtained using the ET 16-plex with as few as 28 cycles and a 97% profile was obtained at 27 cycles. The higher performance of the ET 16-plex was further exhibited when comparing the 27-cycle PCR samples (98% vs. 87% profile). The results of this study demonstrated the potential of using ET-cassette labeled primers to achieve typing of compromised DNA samples without raising the number of PCR cycles, thereby reducing analysis time and complication in DNA profile interpretation.

Real World Samples Typing: To explore the potential of using ET cassette labeled primers to perform analyses on casework samples, we have typed 6 difficult forensic DNA samples from case evidence previously processed by PBSO. The sexual assault case DNA samples were extracted from buccal swabs, semen swab from underwear (both sperm and non-sperm fractions), whole blood and blood swabs from a towel. All DNA samples (0.5 ng) were amplified using the ET 16-plex and PowerPlex 16 systems under the same conditions, except for one sample which had only enough DNA for ET 16-plex analysis. DNA profiles generated on the μCAE system from samples amplified by both 16-loci multiplex kits were compared to the originally reported DNA profiles obtained by PBSO using the 9-loci PowerPlex 1.1 typing system wherever possible.

In one casework sample, DNA profiles could only be obtained using the ET 16-plex for the blood standards of the suspect and the victim as well as a blood swab from a towel. Six and seven alleles were detected from the victim's whole blood (Figure 9A) and towel blood swab (Figure 9B) using the ET 16-plex while zero or one allele was obtained using PowerPlex 16. The partial ET 16-plex blood standard profile provided additional allele information (2 alleles) and hence discrimination power for identification. For the

blood swab sample, since typing using PowerPlex 16 did not generate any profile information, the partial ET 16-plex profile was particularly useful as it offered enough information to link the blood on the towel to the victim, excluding the suspect. The broader ladder peaks compared to Figure 9B are partly due to the smaller time scale used and possibly old polymer and coating, which can be observed with commercial CE systems and is not characteristic of this platform or the chip itself which exhibits an ~90% success rate.

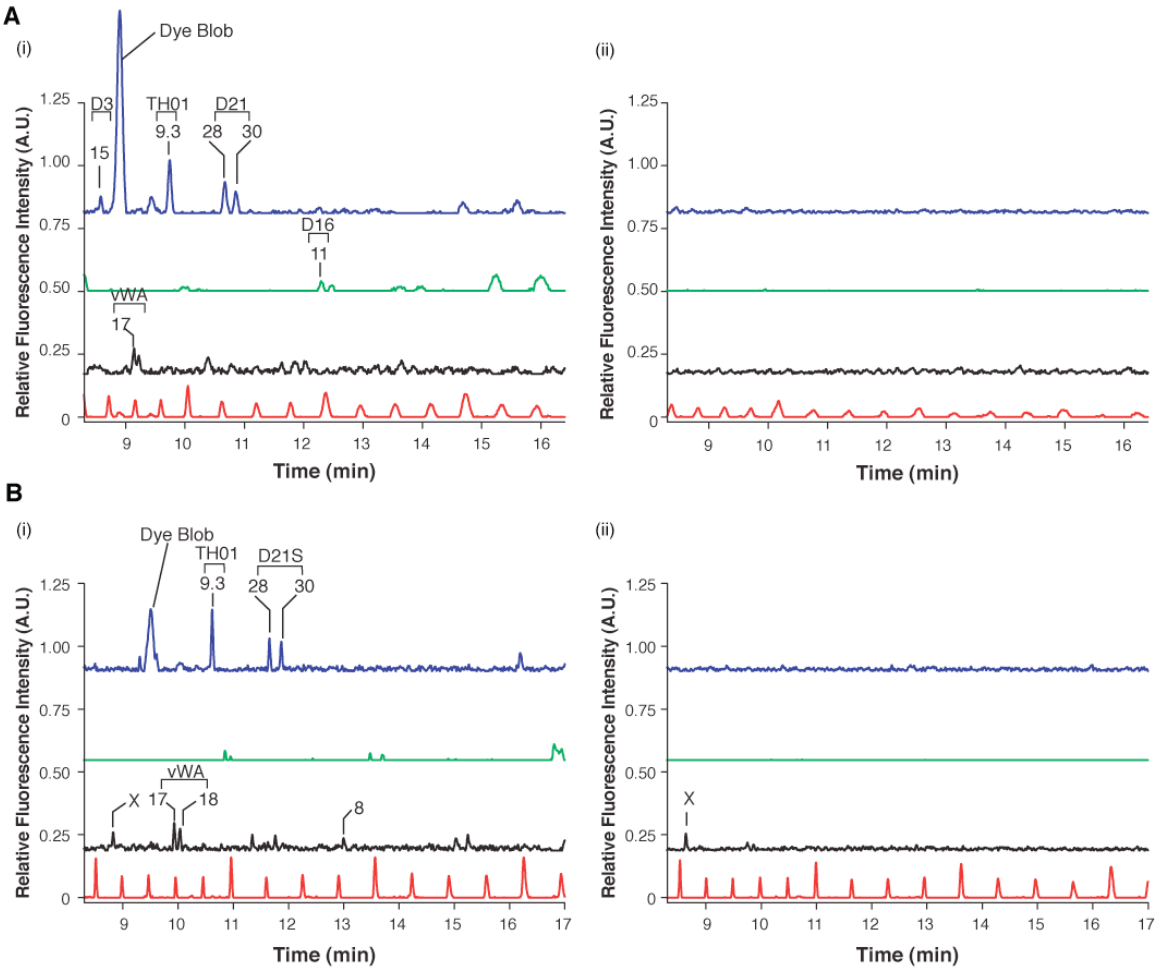

Figure 9. Profiles of closed PBSO casework DNA samples extracted from (A) victim's whole blood and (B) towel swab, both amplified under the same conditions with (i) ET 16-plex and (ii) PowerPlex 16. Partial profiles (6 – 7 alleles) were obtained for samples amplified using the ET 16-plex while only 1 allele or none were typed using PowerPlex 16.

In a second set of casework samples, DNA profiles for the suspect's buccal swab and the non-sperm fraction semen swab samples produced using both multiplex typing systems were comparable. Nonetheless, the non-sperm fraction profile information linked

the underwear back to the victim, who was uncertain about the possession of the underwear and this conclusion could not be drawn on the original report. Due to limited DNA availability, only ET 16-plex profile results were obtained for the sperm fraction of the semen swab sample. Although no comparison could be made to a PowerPlex 16 profile for this sample, the partial ET profile results (6 alleles) provided additional allele information to those obtained by PBSO. The ability to gain additional profile information from previously difficult casework samples demonstrates the importance superior performance of ET labeled primers to type challenging DNA samples commonly encountered in forensic investigation.

The development and demonstration of a multi-color energy-transfer cassette labeled 16-plex DNA typing system that produces higher sensitivity than conventional single-fluorescence dye labels offers a powerful tool for human identification. The facile cassette-primer coupling procedure provides a convenient alternative to new typing system construction. With its higher sensitivity, ET labeled multiplex can generate DNA profiles from minute amount of DNA, which is crucial to forensic investigation. It also allows the use of fewer PCR cycles to reduce the incidence of allele drop-in and false amplifications, producing cleaner DNA profiles for more reliable profile interpretation when typing poor-quality DNA samples. The combination of ET dye labels with μCAE technology presents another advancement to the field of forensics DNA typing.

4. *Development of single channel integrated PCR-CE analysis system on a portable instrument:* We have successfully developed and evaluated a portable forensic analysis system consisting of a microfluidic device for amplification and separation of STR fragments together with an instrument that contains 4-color confocal laser fluorescence detection and all the necessary optical and electronic components for chip operation. To explore the forensic applications of this portable system, a 4-plex mini-Y chromosome STR typing kit was developed and successfully performed on the microsystem with a 90-min analysis time. The performance of this system for forensic STR typing was evaluated on sensitivity, real-case sample and mixture analyses. A field demonstration of the real-time DNA typing was successfully performed. Results of this work and the technical

details were disseminated in the *Analytical Chemistry*[54]. To further realize a practical on-site STR typing, a 9-plex autosomal STR typing system was developed and optimized. We are now exploring the feasibility of using our portable microsystem to perform real-time DNA analysis at a mock crime scene in collaboration with PBSO. Our demonstration of successful STR analyses performed on this portable PCR-CE system validates the concept of point-of-analysis DNA typing of forensic casework, of mass disaster samples, or of individuals at a security checkpoint.

Microdevice design and fabrication: The 1st generation microdevice we developed for 4-plex mini-Y STR typing contains a 160-nL PCR chamber with an integrated heater and a four-point resistance temperature detector (RTD) for PCR amplification and a 7-cm-long CE separation channel for CE separation (Figure 10A). The structure of this microfluidic system is similar to the device developed in our group previously,[54] but the design has been adapted for the portable instrument. The microdevice is constructed with four layers: a glass manifold, a PDMS membrane, a glass heater/channel wafer, and a glass RTD wafer (Figure 10B). The PCR chamber (bottom side of the heater/channel wafer) and the RTD (top side of the RTD wafer) are laid next to each other after bonding. The microfabricated PCR heater is deposited on the top side of the heater/channel wafer and covers the PCR chamber and the RTD to facilitate thermal cycling under the control of the temperature feedback from the RTD. The glass manifold wafer actuates the PDMS microvalves for fluidic control. The integrated PCR heater was re-designed in an iterative process using computational simulation as a guide to create uniform heating over the entire PCR chamber and to facilitate fast thermal response times for high-efficiency and balanced amplification of multiple STR loci. With this new heater, the temperature differences between the center and the edge of the PCR chamber were reduced to less than 1 ^{o}C. The temperature ramp rates can reach 11.5 ^{o}C/s for heating and 4.7 ^{o}C/s for cooling without any active cooling.

To extend the microdevice to autosomal STR typing, a 2nd generation of the PCR-CE microdevice with a co-injection structure has been constructed to achieve sizing calibration. As shown in Figure 10C, a 20-μm wide microchannel connected to a sizing standard reservoir was added to the cross injector. The width ratio of the co-injection channel to the PCR sample channel was optimized to achieve balanced injection of PCR

amplicons and sizing standard simultaneously. Figure 10D shows a fluorescence image of PCR amplicons co-injected with a sizing standard. Since the fluorescence of the sizing standard is relatively weak, only the PCR product can be seen on this image. Nevertheless, it clearly shows that the PCR product encompasses half the sample plug in the cross injector and the sizing standard the other half.

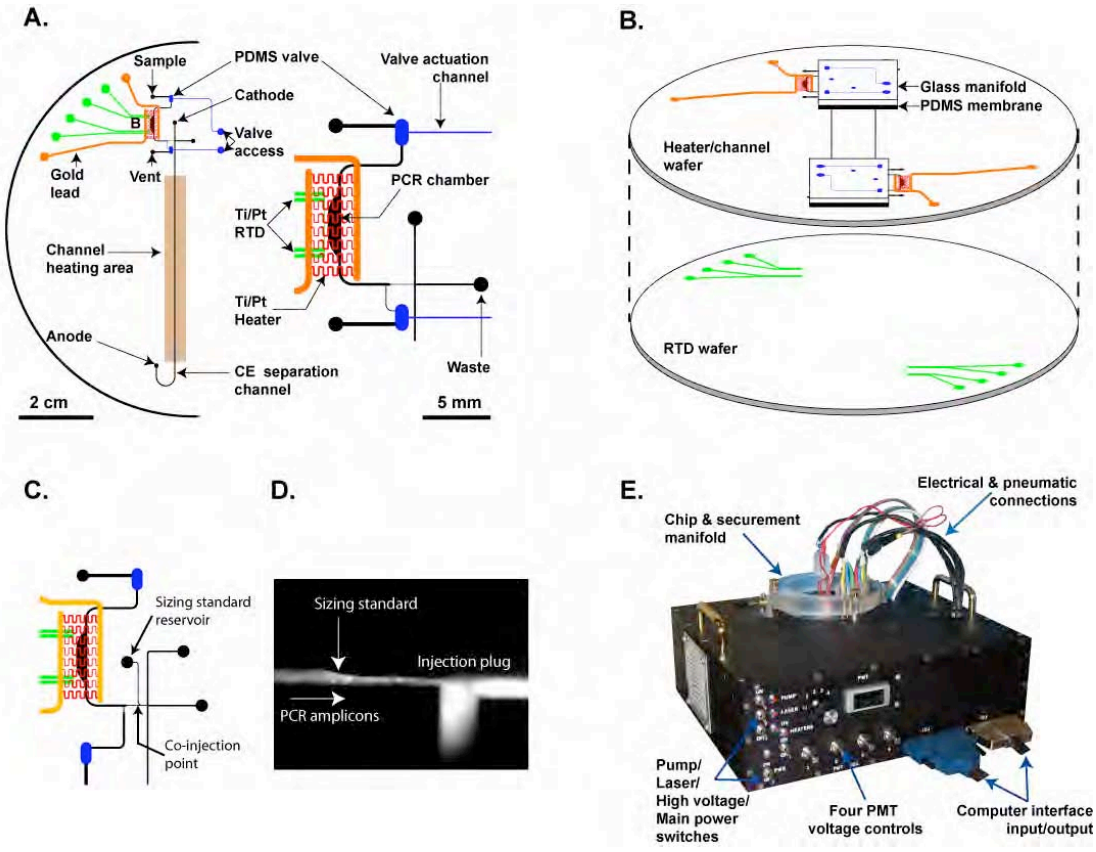

Figure 10. (A) Mask design for the PCR-CE microchip. The glass microchannels are indicated in black, the microfabricated RTD and electrodes are in green, the heater is shown in red, the gold leads of the heater are in gold, and the valves are drawn in blue. (B) Exploded view of the assembly of the PCR-CE microchip, showing the RTDs on the upper surface of the RTD wafer, the glass microchannels etched in the lower surface, and the heaters fabricated on the upper surface of the heater/channel wafer. (C) Design of the PCR-CE chip with a co-injection structure. (D) Fluorescence image of the PCR amplicons and sizing standard co-injection. (E) Photograph of the portable PCR-CE system. The analysis system box has dimensions 12 × 10 × 4 in.

The microfabrication process is similar to that described previously.[55, 56] Briefly, to form the heater/channel wafer, a 550-µm thick D263 glass wafer was coated with 2000-Å

amorphous silicon on one side and 200-Å Ti and 2000-Å Pt on the other side. The channel pattern was photolithographically transferred to the amorphous silicon side, then the sacrificial silicon was etched using SF_6 and the exposed glass was etched to a depth of 38 μm in a 49% hydrofluoric acid bath. The integrated PCR heaters were fabricated on the Ti-Pt side of the same wafer. The gold heater leads (5 μm thick) were patterned and electroplated onto the open Ti-Pt seed layer. Photoresist was then removed and the wafer was repatterned to define the heating elements using an ion beam etching system. Finally, holes were drilled using a CNC mill for via holes, fluidic reservoirs, as well as electrical and pneumatic access holes. The RTD wafer was fabricated on a 700-μm thick D263 glass wafer coated with 200-Å Ti and 2000-Å Pt using similar photolithography and etched using hot aqua regia. The RTD wafer and the heater/channel wafer were thermally bonded in a vacuum furnace at 580 ^{o}C for 6 hr. The glass manifold was fabricated from a 700-μm D263 glass wafer using the same glass etching method described above, and diced into 23 × 18 mm pieces. The microvalves were assembled by cleaning the PDMS membrane in a UV-ozone cleaner for 1 min and then sandwiching the membrane between the bonded wafer stack and the glass manifold. This method results in a tight but reversible glass-PDMS bonding.

Portable instrument: The instrument used to perform analyses with the microdevice is shown in Figure 10E. The instrument contains a 488-nm frequency doubled diode laser, an optical system for detecting four different fluorescence signals, pneumatics for the on-chip PDMS microvalves, electronics for PCR temperature control, and four high voltage power supplies for CE. The weight of the instrument is 10 kg with a power consumption of 20 W, which can be supplied by a car battery. A LabVIEW graphical interface (National Instruments, Austin, TX) developed in-house was used to control the system through two DAQ boards (National Instruments). The detailed information can be found in Appendix A.

(i) Quadruplex mini-Y STR typing: The quadruplex mini-Y STR system included amelogenin and three Y-chromosome STR loci, DYS390, DYS393 and DYS439. The forward primers were labeled with energy transfer (ET) dye cassettes developed in our group. The 20-μL PCR mixture prepared for each experiment was comprised of Gold

ST*R buffer (Promega), templates ranging from 0-50 copies in the 160-nL PCR chamber, primers, and FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). The thermal cycling protocol begins with the activation of the Taq polymerase at 95 ^{o}C for 4 min, followed by 35 PCR cycles of 95 ^{o}C for 10 s, 58 ^{o}C for 60 s, 72 ^{o}C for 30 s, and a final extension step for 2 min at 72 ^{o}C. The total PCR time is 64 min.

Linear polyacrylamide (LPA) (5% w/v) with 6 M urea in 1×Tris TAPS EDTA (TTE) buffer was firstly loaded into the entire CE separation system. PCR mixture in the sample reservoir was then moved into the PCR chamber by vacuum applied at the vent reservoir. After PCR, the CE channel was heated to 70 ^{o}C using the channel heater. After opening the microvalve adjacent to the sample reservoir, the amplified sample was electrophoretically injected into the CE channel towards the waste by applying an electric field of ~100 V/cm while floating the anode and cathode. A separation field of 250 V/cm was then applied between the cathode and anode, and a 5-s backbias was applied to the sample and waste at 80 V/cm, and floated for the rest of the separation. After each run, the glass manifold was removed, the PDMS membrane was replaced, and channels and chambers were cleaned completely using piranha solution (7:3 H_2SO_4: H_2O_2). Data processing procedures include baseline adjustment, fluorescence cross-talk analysis and convolution filtering in BaseFinder 4.0 program. The key findings are summarized below.

Monoplex and multiplex amplifications: We first performed singleplex amplifications on each locus to examine the functionality of the PCR-CE microsystem as well as the amplification performance of these DNA markers. In these PCR experiments, each DNA marker was amplified from 20 copies of 9948 male standard genomic DNA with 32 PCR cycles. As shown in Figure 11A, each DNA marker demonstrated a similar amplification efficiency and good sensitivity. Following successful amplifications on each locus individually, a multiplex PCR-CE experiment was carried out on this four-locus multiplex system. Starting template (50 copies of 9948 male standard DNA) was loaded in the PCR chamber and 35 PCR cycles were performed. As shown in Figure 11A, all the peaks (106, 112, 123, 171 and 191 bp) were fully resolved and balanced. Compared with singleplex amplifications, multiplex STR amplifications exhibit lower amplicon yields due to competition between each locus. Therefore, both the initial

template copy number (50 copies) and the PCR cycle number (35 cycles) were increased to compensate for this effect.

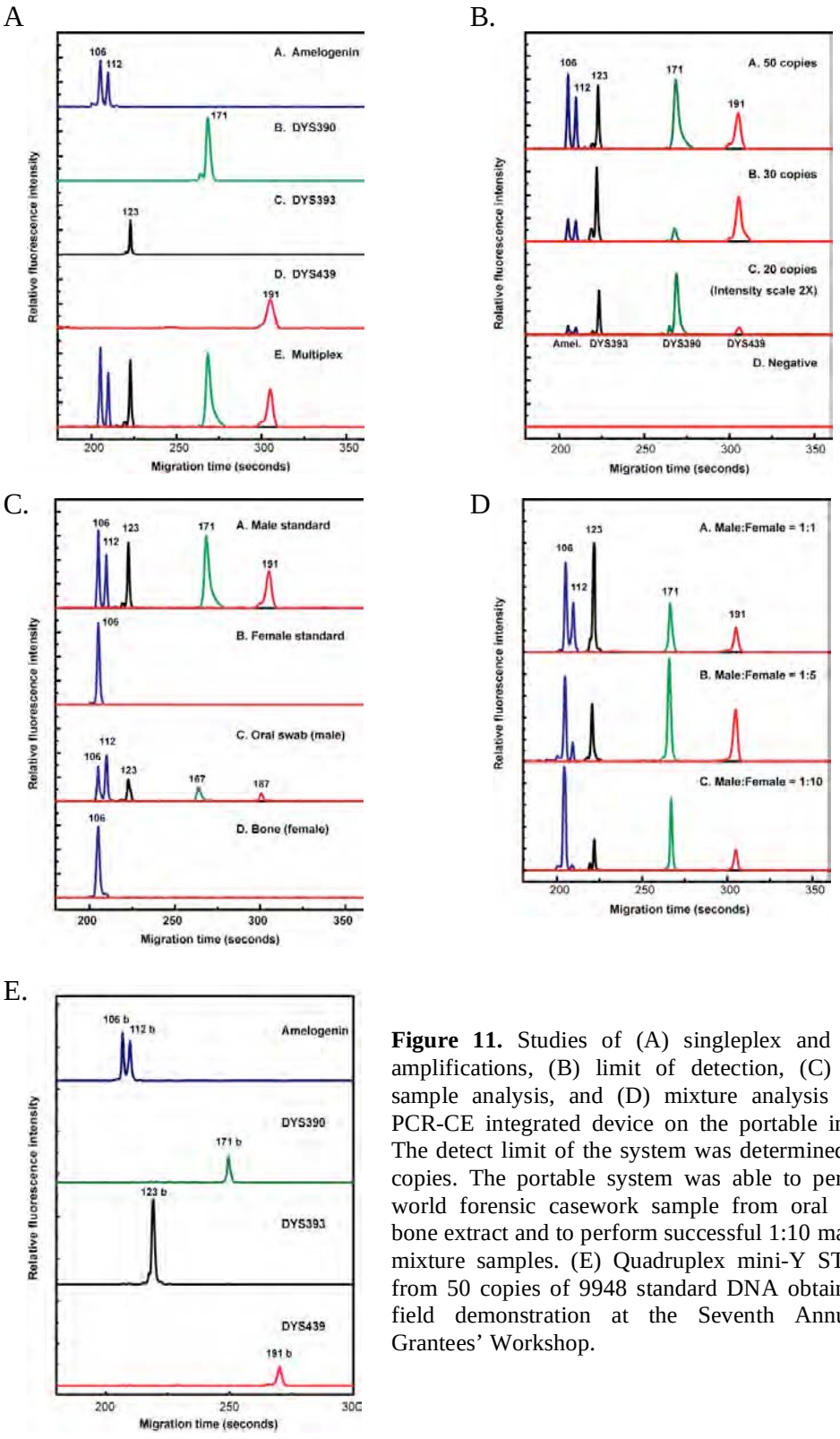

Figure 11. Studies of (A) singleplex and multiplex amplifications, (B) limit of detection, (C) casework sample analysis, and (D) mixture analysis using the PCR-CE integrated device on the portable instrument. The detect limit of the system was determined to be 20 copies. The portable system was able to perform real world forensic casework sample from oral swab and bone extract and to perform successful 1:10 male-female mixture samples. (E) Quadruplex mini-Y STR profile from 50 copies of 9948 standard DNA obtained in the field demonstration at the Seventh Annual DNA Grantees' Workshop.

A limit-of-detection (LOD) analysis for multiplex amplifications of 9948 male standard genomic DNA was performed. Figure 11B presents results from a series of amplifications conducted from 0, 20, 30 and 50 copies of template in the PCR chamber with 35 PCR cycles. Even with only 20 copies of DNA template in the reactor, the multiplex amplification still shows all the expected peaks in the electrophoregram. An amplification from 10 copies was also performed, however, a complete profile was not obtained. Finally, it should be noted that the absence of any amplicons in the negative control (0 initial copies) demonstrates the effectiveness of the piranha cleaning conducted after each run.

Analysis of forensic casework samples: To verify the performance of our portable system on real-world forensic samples, we selected two typical samples, one from an oral swab and the other from human bone, which were previously processed and analyzed by the Palm Beach County Sheriff's Office. Figure 11C (Trace A and B) presents the PCR analyses conducted from male and female standard DNA as controls, showing all the expected peaks with correct gender discrimination. Figure 11C (Trace C) presents an amplification and analysis of the DNA sample extracted from an oral swab. All the amplicons in four loci were successfully obtained, indicating the sample is male DNA. Figure 11C (Trace D) shows only one peak at 106 bp, corresponding to the successful amplification of female human bone DNA.

Mixture analysis: Mixture analysis was carried out by mixing male and female standard genomic DNA together during the sample preparation. The male DNA in each run was maintained at 50 copies, while the female DNA was increased to achieve ratios of male-to-female genomic DNA of 1:1, 1:5 and 1:10, respectively, resulting in ratios of Y-to-X chromosomes of 1:3, 1:11 and 1:21. The results of this experiment in Figure 11D show that, as the ratio increased, the 106-bp amplicon from X chromosome became more and more dominant over the 112-bp Y-chromosome product. The peak area ratios are roughly equal to the initial template ratios of Y-to-X chromosomes (1:3, 1:11 and 1:21).

The other three Y-chromosome loci (DYS390, DYS393 and DYS439) were still fully amplified and balanced in each case. However, slight signal reductions were observed, due largely to the increase of the 106-bp X-chromosome amplicon which used up most of the PCR resources.

Field demonstration: Finally, we carried out a field demonstration of the portable microsystem at the Seventh Annual DNA Grantees' Workshop at Washington, D.C. in 2006. As shown in Figure 11E, a full profile of the quadruplex STR system amplified from 50 copies of 9948 male standard DNA was obtained, demonstrating the portability, robustness and reliability of the system.

(ii) Nine-plex autosomal STR typing: To further extend the application of this portable microsystem, a 9-plex autosomal STR typing system has been constructed using primer sequences employed in PowerPlex® 16. It consists of Amelogenin, and eight STR loci (D3S1358, THO1, D21S11, D5S818, D13S317, D7S820, vWA, and D8S1179) with a size range of 106–258 bp. The on-chip thermal cycling protocol is the same as those in the Promega PowerPlex® 16 Technical Manual, except that the Taq activation time, the extension holding time in each cycle, and the post extension time were reduced to 4 min, 30 s, and 10 min, respectively. Therefore, the total PCR time was shortened to 2 hr. To achieve sizing calibration, a sizing standard (MegaBACE ET550-R, GE Healthcare) was loaded into the sizing standard reservoir after the thermal cycling. During the sample injection for CE separation, same voltage was applied on the sample and sizing standard reservoirs to inject the PCR products and sizing standard into the injection channel. The four-color fluorescence data recorded by the portable instrument were first converted to binary format and appended with proper header information by a custom LabVIEW program (National Instruments). The pre-processed data files were then input into MegaBACE Fragment Profiler 1.2 (Amersham Biosciences) for allele calling. The key findings were summarized in the following:

System characterization: We first investigated on-chip PCR amplification and separation of 9947A and 9948 standard DNA using the 9-plex STR system on the 2nd generation microdevice. Figure 12 shows the successful amplification profiles on 9947A and 9948 standard DNA obtained from 100 copies of template with 32 PCR cycles in 2.5

hr. using this PCR-CE microdevice. Sizing standard ET 550-R was co-injected with PCR products in each run to facilitate the size calculation of alleles. All the alleles of 9947A and 9948 standard DNA were balanced, fully resolved, and correctly sized. To further evaluate the performance and reproducibility of the portable instrument, three independent analyses on each sample with 100 copies of starting template were performed. Table 4 lists the average fragment sizes and the standard deviations (<0.5 bp) of all the alleles (except for D13S), demonstrating the good performance of the co-injection structure. While there is some loss of resolution due to the short separation channel (7 cm), we found that this channel length is sufficient for fragment size of 100-250 bp for initial human identification purpose. Further improvements can be easily made by increasing the channel length and optimizing the separation conditions.

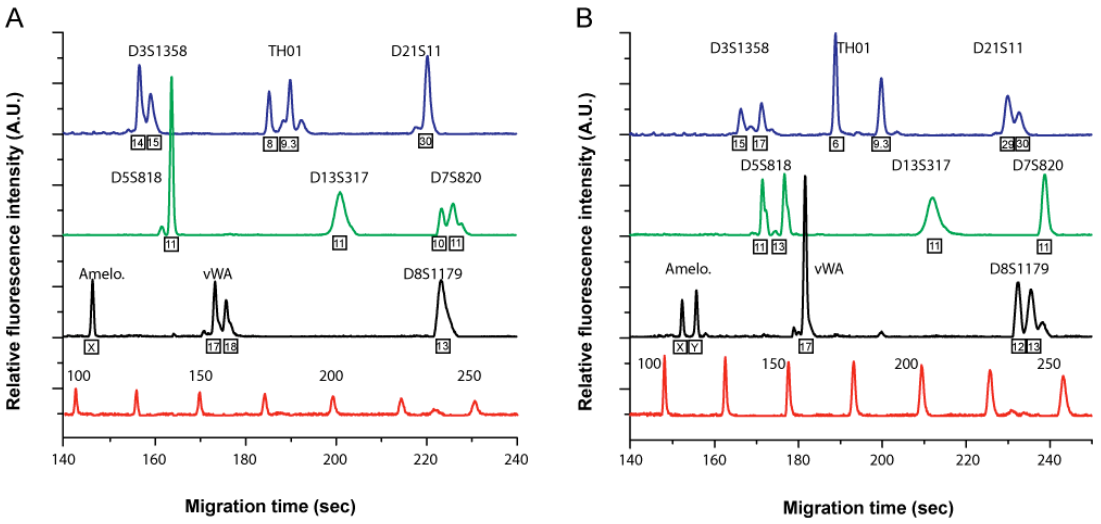

Figure 12. 9-plex STR profiles obtained from 100 copies of (A) 9947A and (B) 9948 DNA on the portable PCR-CE microsystem with 32 PCR cycles. Separation was achieved in 5 % LPA with 6 M urea in a 7-cm microchannel at 250 V/cm.

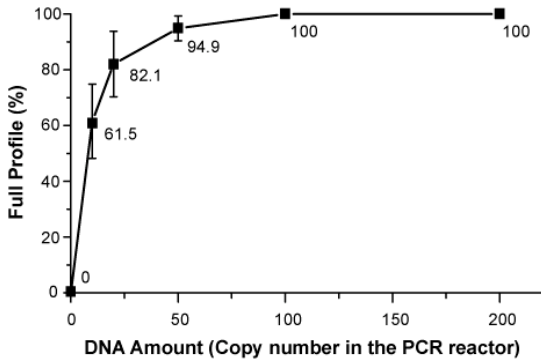

Figure 13. Percent of the full 9-plex STR profiles obtained on the portable microsystem as a function of input genomic DNA.

A limit-of-detection (LOD) analysis for multiplex amplifications of 9947A standard DNA was also performed with serially diluted DNA templates (200, 100, 50, 20, 10 and 0 copies). Figure 13 shows that the portable microsystem was able to produce 100% profiles down to 100 copies of DNA in the PCR reactor. Even with only 10 copies of DNA template, the multiplex amplification still showed 61.5% of the total expected peaks (average 8 in 13 expected peaks) in the electrophoregram, which may provide useful information to forensic investigation. Additionally, the negative control (0 initial copies) shows no amplicons, demonstrating the absence of carry-over and contamination.

Table 4. Average fragment sizes for standard DNA samples using coinjector structure

	9947A Female Standard DNA		9948 Male Standard DNA	
	Allele: Average Size (bp)	Standard Deviation (bp)	Allele: Average Size (bp)	Standard Deviation (bp)
D3S1358	14:126.5	0.26	15:131.2	0.06
	15: 131.0	0.23	17: 139.2	0.10
THO1	8: 177.3	0.35	6: 168.2	0.26
	9.3: 185.2	0.46	9.3: 185.3	0.42
D21S11	30: 234.8	0.40	29: 231.2	0.17
			30: 235.0	0.15
D5S818	11:139.4	0.49	11:139.8	0.06
			13: 148.3	0.06
D13S317	11: 204.0	0.80	11: 203.9	0.21
D7S820	10: 239.7	0.26	11: 243.9	0.12
	11: 243.5	0.35		
Amelogenin	X: 107.3	0.06	X: 107.3	0.12
			Y: 113.2	0.10
vWA	17:156.4	0.10	17: 156.4	0.12
	18: 160.7	0.12		
D8S1179	13: 238.7	0.40	12: 234.7	0.06
			13: 239.0	0.10

Evaluation of DNA extraction: DNA extraction is critical for performing real-time STR typing using the portable microsystem. The extraction method should be compatible with on-site rapid analysis, and should provide sufficient DNA in quality and quantity. We evaluated four DNA extraction methods (Maxwell® 16 system, DNA-IQ™, QIAamp™, and Ultraclean™) in collaboration with PBSO. DNA samples were extracted from blood on swabs by PBSO using these DNA extraction methods according to manufacturer's protocols. Then these samples were shipped to Berkeley to run on the

portable instrument. As shown in Figure 14, all the extraction methods yielded DNA samples with reasonable quality and quantity for full STR profiles on our PCR-CE microdevice. After carefully exam their performance and operation, we finally chose Maxwell® 16 system as our on-site DNA extraction method, since it provides more reproducible DNA samples.

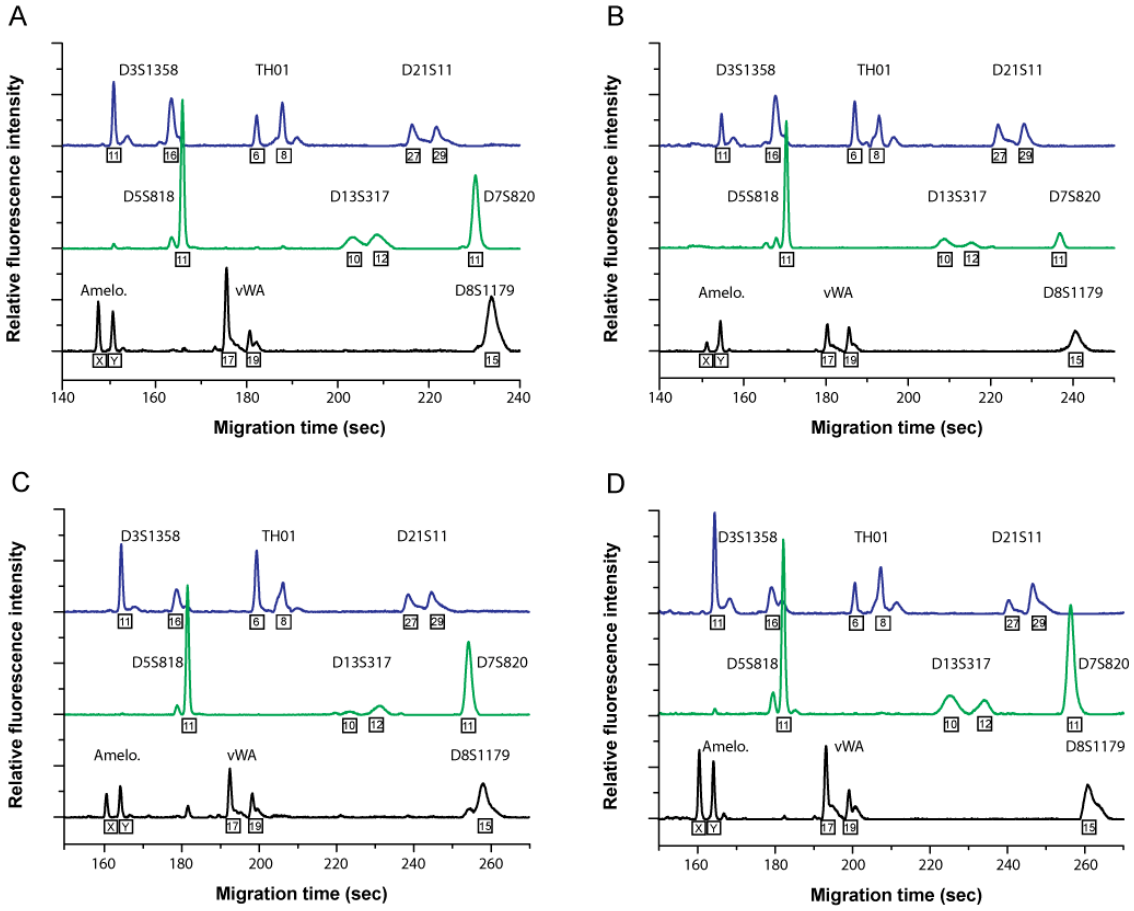

Figure 14. Evaluation on DNA extraction methods. Blood-on-swab samples were processed using (A) Maxwell® 16 system, (B) DNA-IQ™, (C) QIAamp™, and (D) Ultraclean™. Each extracted DNA (100 copies) was successfully typed with similar performance on the portable instrument.

Simulated casework sample analysis: We also explored the ability of our system to analyze samples from commonly encountered stain sources in forensic investigations. The simulated casework samples were prepared as following: 30-μL blood for each stain

was dropped on a piece of cloth and a magazine surface. After air drying, these stains were wiped with swabs. Then, these swabs were processed and DNA was extracted following the Maxwell 16 system protocol. The extracted DNA samples were analyzed on the portable microsystem with about 300 copies of template. Figure 15 shows that both stains processed using Maxwell system can provide enough DNA for our system to produce complete and balanced 9-plex STR profiles. All the alleles were correctly typed and confirmed PBSO using PowerPlex 16 BIO. This work establishes the protocols

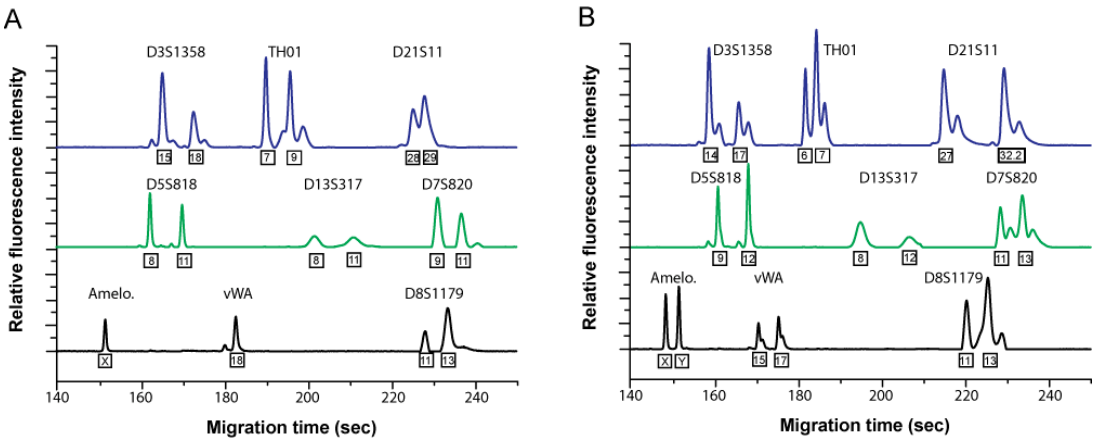

Figure 15. 9-plex STR analysis on simulated casework samples extracted from stains on (A) cloth and (B) magazine using Maxwell system. In each case, 30 starting template copies were amplified and typed on the portable instrument.

that will be used in the future to perform a real-time forensic analysis at a mock crime scene in collaboration with PBSO.

5. Integration of capillary electrophoresis with sample cleanup: Allele or locus drop-out of the larger loci is often encountered when typing challenging DNA samples. This loss can be due to insufficient amplification during PCR, and/or inefficient injection during CE. We have previously demonstrated the power of integrating sample purification with DNA separation for sequencing applications.[29-32] This technology has been successfully demonstrated on-chip to pre-concentrate and clean-up DNA sequencing reactions by employing a capture gel matrix containing a short oligo sequence complementary to a sequence found among all amplified sequencing reaction products.[29] A similar approach should also be valuable for forensic DNA typing.[58] We began the

transfer of this technology to forensics by designing short DNA capture probes targeted to internal but nonrepeating sequences in each of the sets of STR products. We selected the primer sequences used in PowerPlex 16 (as well as in our ET multiplex kit) to facilitate this capture strategy. The fundamental advantage of this approach is that by selecting the mole fraction of the various capture oligonucleotides, the contribution of the various captured fragments can be normalized to make peak heights more uniform and the improved injection efficiency will enhance LCN and degraded DNA typing.

We designed 16 capture oligos to have a melting temperature (T_m) of ~ 55 °C to facilitate a target capture (hybridization) temperature of 50 °C. A series of cross-hybridization checks using commercial software were performed comparing the capture oligo sequences with themselves as well as with the primer and the products of different loci to ensure specificity prior to syntheses of the capture gel. Our initial evaluation of capture temperature was performed for a limited multiplex with the focus on optimization of the basic procedures, chip and process design on a microdevice containing individual capture reactors.

Since capture oligo technology was originally developed for ssDNA sequencing reaction cleanup while the STR reaction produces dsDNA, we also investigated the possibility of generating ssDNA for forensic STR amplifications using locked nucleic acid (LNA) modified primers which allow higher T_m's without adjusting the primer sequence. In this approach, a lower annealing temperature was used in the first series of PCR cycles for dsDNA generation followed with a higher annealing temperature for the later series of PCR cycles. By this process only LNA-primers would bind to the templates at the higher temperatures to generate primarily ssDNA products. Dr. George Sensabaugh's student Sandy Calloway, supported by this grant, worked with Stephanie Yeung to perform studies of the annealing temperatures and primer concentrations in monoplex reactions using LNA-modified primers. The results of the studies showed low efficiency of ssDNA generation, an obstacle in developing a multiplex system with LNA-primers. We therefore discarded this strategy and reverted to the capture of ssDNA from thermally denatured dsDNA products.

A bench-top capture system was constructed to serve as an emulation of capturing real PCR reactions while allowing us to image the capture process easily using sample

with sufficient fluorescence intensity. The system consisted of a thermal cycler for temperature control, a power supply for voltage control, and a inverted U-shape glass capillary tube (1.1 mm I.D., 1.3 mm O.D., 5 cm length) with each end inserted inside a 600-μL microfuge tube, one containing amplified PCR products and the other containing running buffer. Capture gel matrix was loaded into the glass tube prior to capture. Dr. George Sensabaugh's student Jessica James, supported by this grant, worked with Stephanie Yeung to investigate the feasibility of this device and to characterize the optimal capture conditions of both fluorescently labeled ssDNA oligos complementary to capture probe sequence as well as dsDNA from STR reactions. However, there were two major drawbacks of this system — the large capture oligo consumption due to the microliter-volume glass tube as well as the relatively large elution volume required in the microfuge tubes resulted in low-concentration elution products for detection. We therefore switched to an alternative microfluidic approach.

Recently, our group has developed a nanoliter-scale microdevice that integrates the three Sanger sequencing steps[31] as well as a 4-lane integrated PCR-CE array microdevice to amplify femtogram amounts of dsDNA followed by electrophoretic separation in less than 30 min.[33] We have also perfected an even more efficient direct injection method that permits nearly quantitative injection of the selected fragments.[57] By adopting this inline injector geometry, which eliminates the delicate cross-injector timing, we expect a decadic improvement in sample injection efficiency and also dramatically improved reliability for microchip CE analysis. This method allows sample desalting and concentration in a single step and precise quantitation of the captured samples. At the same time, we have conducted preliminary studies on capture efficiency as a function of the target capture sequence locations within dsDNA products. Results of this study indicated that capture efficiency decreases as the target capture sequence is shifted further from the ends of the dsDNA. In DNA sequencing, the design of capture oligo targets a universal sequence between the primer and the unknown sequences. In STR typing, however, the entire amplicon sequence, except for the number of repeats, is well known and should therefore allow better capture efficiency with a capture probe targeting the end product sequence.

We are now combing the integrated capture cleanup-μCE device and the better inline injector geometry together with the alternative end-capture oligo design for STR product cleanup and typing. Note that the current integrated capture-CE microdevice relies only on the detection of simple DNA fragments during the very last separation step, while leaving the behavior of dsDNA products in the upstream processes uncharacterized. However, from our previous experiments, we have determined that a higher-sensitivity imaging system that will allow us to visualize the capture-CE interface process will be critical to understanding the physical and chemical phenomena that determine the efficiency of our capture system. Such knowledge will help us accelerate towards our end goal by shortening the amount of time and effort in troubleshooting an integrated process, especially for complicated STR samples. After the completion of ET-multiplex development, Stephanie Yeung is now focusing on setting up a higher-sensitivity imaging system and apply it for on-chip STR sample cleanup and separation. At the same time, a rotation student Nadia Del Bueno has also been investigating the possibility of using photopolymerizable capture gel to precisely define the captured DNA sample plug for improved peak resolution and morphology. Once the most efficient capture-CE conditions are defined, monoplex and limited multiplex STR reactions will be tested and their sensitivity characterized on a single-channel device. Once perfected this technology will be integrated into a multi-channel platform for our 2nd generation bench-top rotary fluorescence scanner.

6. *Development of the 2nd generation forensic scanner:* A 2nd generation bench-top rotary μCAE scanner with reduced size and operational complexity and enhanced capabilities has also been designed. This scanner will have a footprint of ~12”x 12”x 10” and feature an integrated 488-nm laser, temperature-controlled microchip stage, a rotary scanning objective, all the electronics (such as high voltage power supplies, PMT and software interface), optics and fluidic controls. This system will have the flexibility of accommodating one 150-mm diameter wafer in different throughput (12, 24, 48 or 96 channel) configurations. This rotary scanner will be built to accommodate four or more fluorescence color detection in order to suit the increasing need of additional colors for higher discrimination in future STR typing systems. This system will be capable of performing μCAE analysis alone, like the current system installed in VDFS, cleanup and

injection of separately amplified samples, and eventually high-throughput integrated PCR - capture cleanup - CE and other integrated steps.

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Appendix Contents

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MICROCHIP

Microchip Capillary Electrophoresis: Progress Toward an Integrated Forensic Analysis System

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The integration of PCR with capillary electrophoresis on a microchip is undeniably a significant step toward a total integrated forensic analysis system.

INTRODUCTION

Integration of capillary electrophoresis (CE) onto a microchip for forensic short tandem repeat (STR) analysis is the first critical step to produce a fully integrated and automated STR analysis system. Microchip capillary array electrophoresis (μCAE) analyzers provide rapid high-throughput separation of forensic samples and can increase workflow and reduce costs (1). The microchip CE format is also important because it facilitates electrophoretic analysis of submicroliter to nanoliter sample volumes. This low-volume analysis capability facilitates integration of PCR on the microchip, which will further increase automation, improve reliability and reduce operator intervention (2,3). Ultimately such PCR-CE technology also should be integrated with DNA extraction, STR sample cleanup and desalting (4) to make a fully integrated forensic analysis system for both high-throughput work and point-of-analysis applications. The goal of this review is to describe the current capabilities of microchip CE technology and point the way to the future.

MICROCHIP CAPILLARY ELECTROPHORESIS

The advent of microchip-based CE separations of DNA can be traced back more than a decade to a number of laboratories engaged in the effort (5,6). These microchips consist of a glass wafer that has been chemically etched through a photolithographic pattern to define the injection and separation channels. The etched wafer is then bonded to a second wafer containing drilled holes to provide fluidic access to the channels (Figure 1). The transition from conventional glass capillary systems used in DNA sequencing to an etched glass plate demanded that obstacles be surmounted, including the development of 1) reliable cross-injection designs and methods on capillary chips, 2) new separation matrices that provide single-base resolution and are easily pumped into the microchip channels, and 3) novel turn geometries to increase capillary length with no loss in resolution. Such improvements allowed the development of dense microfluidic circuitry while keeping the microchip similar in size to a compact disc (1).

Work in the Mathies' lab at the University of California, Berkeley, and at the Virginia Department of Forensic Science (VDFS) has demonstrated that this microchip system, together with the rotary confocal scanner developed by Scherer *et al.* at Berkeley, produces rapid reliable state-of-the-art forensic analyses. The fast heat dissipation enabled by the high surface-to-volume ratios of microCE (μCE) channels allows high-voltage separation of the nanoliter DNA sample plug. By pairing this virtue with a high-performance sieving matrix, such as linear polyacrylamide (LPA), rapid 20-minute CE with single-base resolution can be achieved. Yeung *et al.* (7) accurately profiled nonprobative and mock forensic samples in <30 minutes using a 96-capillary μCAE device (Figure 2). Data generated by Yeung *et al.* were comparable in quality to commercial CE systems. Moreover, a similar system was successfully

MICROCHIP

implemented at the VDFS as a collaborative effort between the Mathies' lab at UC Berkeley, VDFS and Palm Beach County Sheriff's Office (manuscript submitted).

MICROCHIP PCR

The versatility of PCR in genetic analysis has attracted interest in miniaturization and integration with microchip CE analysis for applications ranging from genotyping for disease diagnostics to forensic DNA profiling. Standalone microchip PCR reactors were initially demonstrated in stationary fluid cycling (8,9) and continuous-flow systems (10,11). To fully realize the potential of microchip PCR, however, it must be integrated with other upstream or downstream analysis steps, such as CE. The first demonstration of coupling microchip PCR with µCE analysis was performed in the Mathies' lab in 1996 (2). This was followed by incorporation of polydimethylsiloxane (PDMS) membrane pneumatically actuated valves and vents for fluidic control and a 280 nl PCR chamber to achieve 20 PCR cycles in only 10 minutes (3; Figure 3, Panel A). This integrated format was recently scaled to multiple reactors on the same chip and applied to genotyping, infectious disease detection and expression monitoring (12). The capabilities of rapid thermal cycling and electrophoresis as a result of fast heat dissipation were critical to shortening analysis time. The precise positioning of tiny heating elements and sensors on a microchip makes temperature control and monitoring more accurate. More importantly, the nanoliter PCR reactor reduces the consumption of expensive PCR reagents, decreasing cost while minimizing pipetting errors between the two steps. The integration of PCR with CE is undeniably a significant step toward a total integrated forensic analysis system.

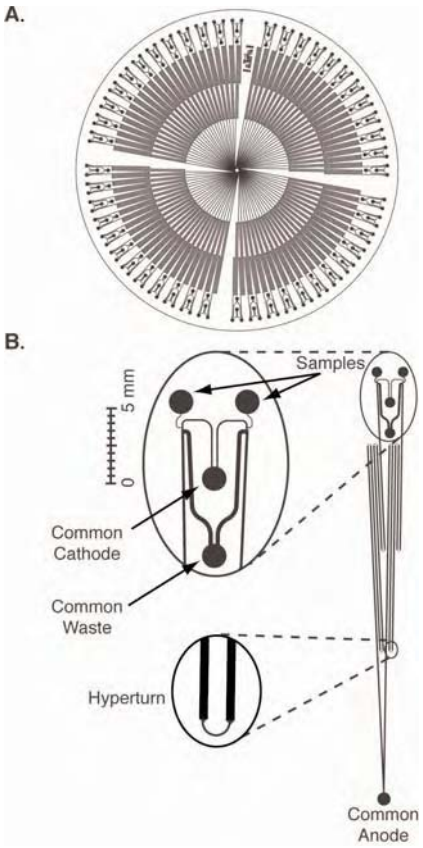

Figure 1. Panel A. Schematic of the 96-channel microfabricated capillary array electrophoresis chip. **Panel B.** Each doublet includes an injector with two sample wells that share common cathode and waste wells. The sample plug is formed at the intersection of the sample and waste arms and electrophoretically migrates toward the central anode, where it is detected by the confocal fluorescence scanner.

PORTABLE ANALYSIS SYSTEMS

There is increasing interest in portable point-of-analysis forensic STR typing systems for military, antiterrorism and mass disaster applications as well as limited crime scene processing (13,14). Toward this end, Liu and co-workers demonstrated STR typing of forensic DNA samples on a portable briefcase-sized device (Figure 3, Panels B and C) that integrates PCR, CE, and fluorescence excitation and detection (13). This system produces a multiplex Y-STR DNA profile from a sample in only 1.5 hours. Figure 4 presents STR results from a real-time demonstration of integrated microchip PCR-CE on a benchtop detection unit at the National Institute of Justice's Grantees' conference in July of 2006, where a DNA sample was profiled during the poster session (15).

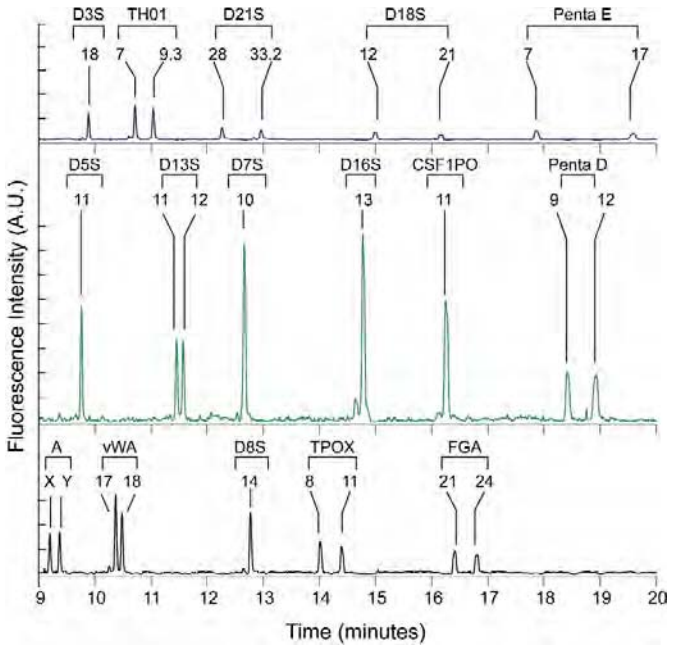

Figure 2. Nonprobative sexual assault casework sample profile generated with the µCAE. PowerPlex® 16 electropherogram showing results from the sperm fraction.

FUTURE DIRECTIONS

What do these technological advances mean for the future of forensic DNA profiling? Rapid, integrated sample analysis systems may create a flexible, dynamic and much more active role for the forensic laboratory. Reaping the greatest benefit requires the concomitant pairing of microchip technologies and expert systems for extremely rapid sample profiling.

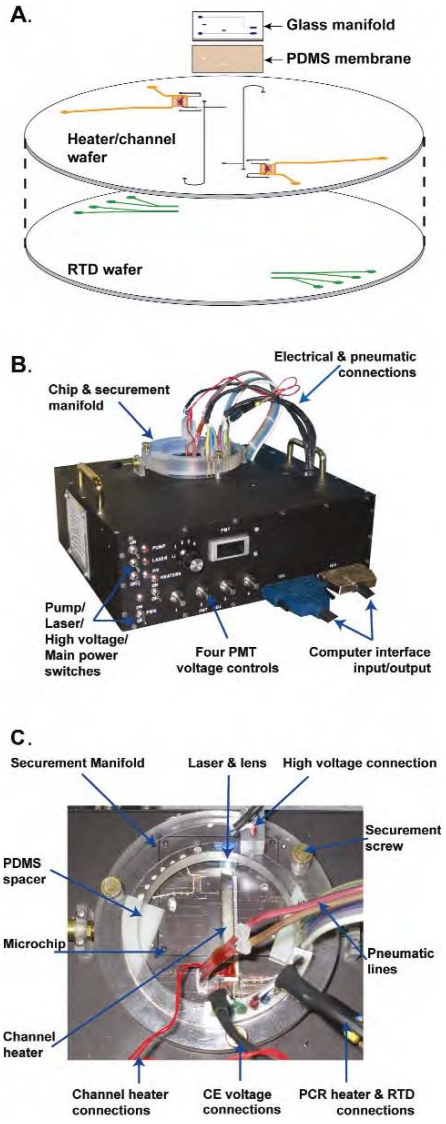

Figure 3. Panel A. Design of the integrated PCR-CE microchip. **Panel B.** Photograph of the portable PCR-CE system. The analysis system has dimensions of 12 × 10 × 4 inches. **Panel C.** Close-up of the microchip and manifold.

Ultimately we envision a microchip system that incorporates DNA extraction from a raw sample, as well as improved processes for STR product cleanup and concentration normalization. Development is in progress to integrate microchip CE with affinity gel capture-based PCR sample cleanup, as recently demonstrated for sequencing (16). This may facilitate extremely sensitive, low-copy-number (LCN) profiling, eliminating the need for increased PCR cycle number while reducing the incidence of contamination, leading to higher rates of success for samples with <100 pg of DNA (17). Moreover, research is also being conducted on STR profiling using plastic chips, furthering efforts to produce commercially viable microchip systems (18). New separation polymers, such as the thermally controlled "viscosity switching" polymers, may facilitate mobile microchip systems by removing the requirement for high-pressure loading while providing a viscous medium for high-resolution fragment separation (19).

CONCLUSION

While an integrated or modular microchip system capable of rapid DNA extraction, amplification, normalization, fragment separation and data analysis will not relieve the ever-present bottlenecks of evidence examination, presumptive testing, report writing and peer review for complex samples, it may produce an automated system that can seamlessly and rapidly perform DNA analysis tasks. This will greatly reduce turnaround times and backlogs, and enhance forensic capacity without increasing cost and staff requirements.

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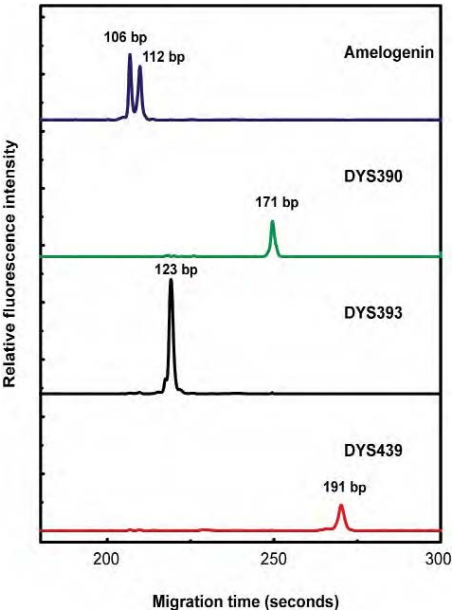

Figure 4. A full 9948 male standard DNA profile obtained from 50 template copies with 35 PCR cycles on the portable PCR-CE integrated system. Presented at the Seventh Annual DNA Grantees' Workshop in Washington, D.C., on June 27, 2006.

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A Forensic Laboratory Tests the Berkeley Microfabricated Capillary Array Electrophoresis Device*

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ABSTRACT

The Berkeley microfabricated capillary array electrophoresis device provided high quality STR profiling using simulated and non-probative forensic samples. A pre-commercial prototype instrument was installed at the Virginia Department of Forensic Science for testing. The successful electrophoresis and short tandem repeat profiling of single source samples and nineteen non-probative casework samples with the PowerPlex® 16 System verified mastery of the device. Sensitivity series and mixture samples were typed with the Powerplex® 16 System and mock sexual assault samples with the PowerPlex® Y System. Instrument performance was assessed as a function of resolution and precision. Resolution measurements were performed using the TH01, CSF1PO, TPOX and Amelogenin loci and precision data collected. Replacement of the Hjerten capillary coating method with a dynamic coating polymer was assessed and ultimately adopted. Successful operation of the μCAE device demonstrates the capacity of this technology to transition out of the research venue and into a practitioner laboratory.

Key Words: Forensic science, microfabricated capillary array electrophoresis, micro-chip, capillary electrophoresis, polyDuramide, PowerPlex® 16, PowerPlex® Y

Technological advancements adopted by forensic science are driven by the need for improved efficiency. While DNA profiling in the forensic arena has undergone dramatic changes in the past two decades, from RFLP to PCR-based hybridization techniques to contemporary short tandem repeat (STR) typing (1,2,3), developments in technology continue to transpire that will ultimately produce dramatic changes in the manner in which DNA testing occurs, specifically in the detection, collection and interpretation of amplified DNA products. Advances in basic research must eventually transition to forensic DNA testing in order to continue moving this important field forward.

Microfabricated and microfluidic chip devices offer much more than a promise of greater speed of forensic analysis. Integration of many of the steps of DNA typing can be realized using the microchip platform, thus making the entire process more automated, more robust and requiring less user manipulation. Microfabricated capillary devices have been successfully integrated with on-chip thermocycling in the research laboratory venue (4-9). This seamless integration not only decreases the reagent volumes required and the overall time consumed for the analysis process, but additionally reduces sample handling, which can eliminate the potential for sample mix-up at those steps and can reduce the risk of laboratory sources of contamination.

Increased speed of fragment separation is better realized using microfabricated capillary array electrophoresis devices (μCAE) than existing commercial capillary systems. While commercial capillary systems, such as the 16 capillary 3130*xl* Genetic Analyzer (Applied Biosystems [ABI], Foster City, CA) and

also the 48 capillary 3730*xl* Genetic Analyzer*,* in use for forensic database samples (ABI), can rapidly achieve fragment separation and detection, the fine tuning capabilities engineered into the microfabricated capillary array electrophoresis process, such as sample plug formation, can enhance resolution while utilizing a shorter capillary length, thereby reducing the time required for fragment separation (10-13). Moreover, advances in novel separation polymer synthesis will generate separation matrices with even greater resolving power that are ideal for microchip applications (14,15). Unique approaches to optimizing capillary electrophoresis on microchip, such as the integrated sample clean-up using affinity gel capture technology, have the potential to enhance the performance of the capillary electrophoresis process in a hands-off, automated fashion (16-18). Additionally, portable capillary electrophoresis microchip instruments have been developed thus providing us a glimpse into potential future directions in which DNA testing may evolve (5,19).

Advances in microfabricated chip technologies are not limited to capillary electrophoresis. Micro-chips capable of DNA purification from samples that may be routinely encountered in a forensic laboratory have been successfully developed (20-22). Many research laboratories are pursuing a total automation system on micro-chip with “sample in, answer out” capabilities.

Ultimately, the technology developed in the basic research laboratory must be transitioned into the setting for which it is intended. Not only does that attest to the question of ease of use, but also provides evidence that the technology in question is not out of reach for scientists not versed specifically in the branch of science that

produced the instrumentation. With that in mind, as part of a collaborative effort between the laboratory of Dr. Richard Mathies at the University of California, Berkeley, the Virginia Department of Forensic Science (VDFS) and the Palm Beach County Sheriff's Office (PBSO), a prototype Microfabricated Capillary Array Electrophoresis device (μCAE) was installed at VDFS for testing in a forensic laboratory by forensic scientists (Figure 1). This study reports our efforts to master the operation of the μCAE device, test its performance using routine validation assessments (concordance, sensitivity, mixture analysis and non-probative sample typing) and evaluate improvements in its routine operation, such as the use of a dynamic coating polymer to coat the glass surface.

MATERIALS AND METHODS

Sample Preparation

DNA samples for the concordance study were prepared from a total of 47 single-source DNA samples obtained from the research laboratory at VDFS, which included buccal swabs and dried blood cards. The 47 samples were extracted and purified manually using the DNA IQ™ System (Promega Corp., Madison, WI) according to the manufacturer's protocol with minor modifications for buccal cell samples and blood stains as described (23) and outlined in the VDFS procedure manual (24) or using an organic extraction procedure, followed by Micron YM-100 clean-up as described (24). The DNA from either buccal swabs or tissue samples was purified for the sensitivity and mixture studies as defined (23) utilizing the robotic DNA IQ™ extraction procedure or using an organic extraction method as

described above. Mock sexual assault samples were provided by Promega Corporation. Twelve samples were created by placing amounts of 1,000 (1K), 10,000 (10K) or 50,000 (50K) spermatozoa on a half-epithelial swab (buccal or vaginal). The mock sexual assault samples were extracted either with the semi-automated Differex™ method according to the vendor's protocol (25) or as described (23).

All samples were quantified with the AluQuant® Human DNA Quantitation System (Promega Corp.), utilizing a Biomek® 2000 Laboratory Automation Workstation (Beckman Coulter, Inc., Fullterton, CA) to set up the enzymatic reaction and the Luminoskan luminometer (Thermoelectron, West Palm Beach, FL) to detect the light signal produced. Samples were quantified with minor modifications from the manufacturer's protocol as outlined in the VDFS procedure manual and as described (24,26). Resulting concentration data were used to dilute the DNA extracts to a concentration of 0.15 ng/µL, for a total of 0.75 ng in the amplification reaction or as indicated.

The sensitivity series were created by placing the indicated quantity of diluted DNA into the amplification reaction. For mixture assays, two purified and quantified DNA samples were mixed together at differing ratios as indicated and placed into the PCR amplification reaction such that the total quantity of DNA amplified was one nanogram (ng).

Non-probative Samples

Nineteen non-probative samples from five different cases were provided by the PBSO laboratory. DNA extracts were quantified at PBSO with Quantifiler™

Human DNA Quantification Kit (ABI), utilizing a Biomek® 2000 Laboratory Automation Workstation (Beckman Coulter, Inc., Fullerton, CA) to set up the qPCR reaction and the ABI 7000 to detect fluorescent signal produced following the manufacturer's recommendations (27) and amplified using the PowerPlex® 16 System following the manufacturer's recommendations (28). Samples were analyzed on both the µCAE device and the ABI 310 and results compared. The data produced from the µCAE device were sent to PBSO for comparison with previous DNA profiling results for the cases.

PCR Amplification – DNA samples were amplified using the PowerPlex® 16 System (PowerPlex® 16) or the PowerPlex® Y System (PowerPlex® Y), both manufactured by Promega Corp., multiplex STR amplification kits as described by the manufacturer (28,29), except for where indicated otherwise, PowerPlex® 16 amplified samples were amplified at half the manufacturer's recommended volume, as described (26). PCR amplification was completed in a GeneAmp System 9600 thermalcycler (ABI).

Separation and Detection – Where indicated, the STR amplicons were electrophoresed on both the ABI Prism® 310 Genetic Analyzer and the µCAE device. Preparation of samples for electrophoresis on the ABI 310 was as follows: 1 µL of each PCR product was added to a loading cocktail containing 24 µL Hi-Di™ Formamide (ABI) and 1 µL Internal Lane Standard 600 (ILS600) (Promega Corp.). One allelic ladder sample was included for approximately every 15 samples. Ladder was prepared in the same manner as PCR products with 1.0 µL PowerPlex® 16 or PowerPlex® Y Allelic Ladder Mix added to the loading cocktail. Samples were

denatured for 3 minutes at 95^{o}C and snap-cooled on ice prior to loading. Electrophoresis and data analysis were performed as recommended (28,29).

Preparation of the samples for electrophoresis on the µCAE was as follows: 1 µl of each PCR product was added to a loading cocktail containing 3 µL Hi-Di™ Formamide, 3 µL sterile Type 1 H_2O, and either 1 or 0.75 µL ILS600 following the procedure used by Yeung *et al* (13). Ladder was prepared by adding 2.0 µL PowerPlex® 16 Allelic Ladder Mix or PowerPlex® Y Allelic Ladder Mix (for PowerPlex® Y amplified samples) to a loading cocktail containing 2.5 µL Hi-Di™ Formamide, 2.5 µL sterile Type 1 H_2O, and either 1 or 0.75 µL ILS600. Samples were denatured for 3 minutes at 95^{o}C and snap-cooled on ice prior to loading.

Instrument Operation and Data Acquisition

For the ABI 310, the operation of the instrument followed the manufacturer's directions for use with the STR typing kits (28,29). The raw data were collected with ABI Data Collection Software and analyzed using GeneScan and Genotyper software, versions 3.1 and 2.5, respectively. Allele calls were performed using the PowerTyper™ 16 or PowerTyper™ Y Macros (Promega Corp.).

Operation of the µCAE device followed the procedures outlined by Yeung *et al* (13). For µCAE chips coated using the modified Hjerten procedure (32), a fresh coating was applied every two weeks as recommended (S. Yeung, personal communication). For µCAE chips coated using the polyDuramide (pDuramide; 13) dynamic coating polymer, the procedure for coating was as follows: µCAE chips were first flushed with deionized sterile water (dH2O) from the central anode to distribute fluid to all capillaries and sample wells, then the chips were filled with 1 M

HCl and allowed to stand for 15 minutes, after which the chips were again flushed with dH2O, followed by filling with the pDuramide and incubation for 15 minutes. After the pDuramide treatment, the chips were flushed with dH2O, then dried and stored until use. The chips were employed for 5 days following coating after which a fresh coating was applied. To setup the chip, a polydimethylsiloxane (PDMS) elastomer ring was placed on top of the cathode and waste wells to create continuous buffer reservoirs. All 96 lanes of the chip were filled simultaneously through the central anode with MegaBACE™ Long Read Matrix linear polyacrylamide (GE Healthcare, Piscataway, NJ) using a high-pressure automatic gel loader. Gel was evacuated from the sample wells and replaced with 2.3 µl of each prepared sample or ladder loading cocktail. The µCAE chip was placed onto the instrument stage heated to 67°C prior to electrophoresis, an electrode array ring containing one electrode pin for each sample well was placed into the sample wells, and a smaller PDMS ring was placed around the central anode. The buffer reservoirs and central anode well were filled with 5X TTE (250 mM Tris, 250 mM TAPS, 5 mM EDTA, pH 8.3) electrophoresis buffer. Sample injection occurred for 55-65 seconds at 170 V while grounding the sample wells and floating the cathode and the central anode wells. The parameters for electrophoresis in the capillary were: a grounded cathode, 2500 V applied to the anode, 200 V to the sample, and 200 V to the waste. Total run time was 28 minutes for the 96 channel chip to electrophorese PowerPlex® PCR products. Although the PowerPlex® Y amplicons were shorter and thus the total time could have been reduced, the same parameters were used for PowerPlex® Y product separation and detection. Following each run,

the chip was placed into an automated high-pressure washer to flush the linear polyacrylamide out of the channels using deionized water. Raw data were collected with a custom LabView program (National Instruments, Austin, TX), the data files baseline corrected and annotated (LabView) appropriately for analysis using Genetic Profiler/Fragment Profiler software (GE Healthcare), then imported into MegaBACE Fragment Profiler v 1.2 software (GE Healthcare) for fragment sizing, color separation and allele designation. Fragment Profiler v 1.2 does not perform peak smoothing of the data.

Appropriate peak filters and bin sets for PowerPlex® 16 and PowerPlex® Y were created using Fragment Profiler (v 1.2). Color separation matrices were also created using the Fragment Profiler software program. In lieu of commercially established threshold settings for peak heights, a signal to noise ratio (S/N) of 3:1 was applied.

Data Analysis for Resolution and Precision studies

Only allelic ladder samples were used for both the precision and resolution calculations. For the ABI 310, two runs containing 15 and 13 allelic ladders, respectively, were completed for a total of 28 samples. Ladders were prepared for electrophoresis in the same manner as described above.

For the µCAE device, a total of 16 and 33 allelic ladder samples were successfully detected using the modified Hjerten and Poly-n-hydroxy-ethylacrylamide (pDuramide) coating procedures, respectively, through a series of four runs for both.

Precision Study – Sizing precision is defined as the ability to reproducibly estimate fragment sizes from run to run on any given instrument (30). Precision was calculated by averaging the standard deviation of size estimates across alleles at each locus. Within-run precision consisted of the standard deviation of size estimates for only those ladders contained within a single electrophoretic run, and between-run precision was calculated by combining data from all runs. All calculations for data from this study, as well as from previously generated data on the μCAE device, were completed in Microsoft (MS) Excel.

Resolution Study – Resolution, defined as the ratio of peak separation to the main peak width, measures the ability of an instrument to separate components (30, 31). A standard chromatographic equation to measure resolution relates the distance between two peaks to the widths of those peaks at half height (Eq 1):

$$R = [2(\ln 2)]^{1/2}(\Delta X)/(W_{h1} + W_{h2}) \quad (1)$$

where ΔX is the peak to peak distance, W_{h1} is the width at half height of peak 1, and W_{h2} is the width at half height of peak 2 (31).

Two different measures of resolution related to this equation, R_b and RSL, were calculated for this study as described by Buel *et al* using both Amelogenin peaks, alleles 7 and 8 in TH01, alleles 9 and 10 in TPOX, and alleles 10 and 11 at CSF1PO (31).

R_b, or base resolution, gives the value of resolution in bases (Eq 2):

$$R_b = \Delta M/R \quad (2)$$

where ΔM is the distance between two peaks in bases (31).

RSL, or resolution length, is an alternative measurement which evaluates a single peak (Eq 3):

$$RSL = W_h/(\Delta X/\Delta M) \quad (3)$$

where W_h is the peak width at half height for the peak of interest, ΔX is the distance between this peak and an adjacent peak, and ΔM is the difference between the two peaks in bases (31).

A fourth resolution measurement, valley value (V_v), is an assessment of the resolution between peaks that differ in length by a single base (31), and was calculated for the 9.3 and 10 alleles of TH01 only (Eq 4):

$$V_v = V/H \quad (4)$$

where V is equal to the height of the valley, or the point where the two peaks merge, and H is the peak height of the larger peak.

Measurements of peak widths, heights, and distances between peaks for data obtained in this study, as well as for data from the µCAE device previously generated at UC Berkeley, were obtained using calipers on printed electropherograms as described in Buel *et al* (31). Distances between peaks in bases were obtained from the appropriate Genotyper or Fragment Profiler software. All calculations were completed in MS Excel.

RESULTS

Concordance Study

Once successful operation of the µCAE had been established at VDFS, 47 single-source samples were amplified with PowerPlex® 16, electrophoresed and

analyzed. Allele calls were compared with the VDFS staff DNA index, typed using PowerPlex® 16 BIO System as described (26,35) and PowerPlex® 16 profiles obtained from the same amplified samples analyzed using the ABI 310. All profiles obtained using the µCAE were consistent with those generated using the ABI 310 and also with the VDFS staff DNA index (data not shown).

Use of Hjerten and pDuramide Coating Procedures

The Hjerten coating of glass capillaries involves the covalent, silanol mediated bonding of acrylamide to the sides of the glass capillary wall (32). This prevents electroosmotic flow (EOF) as well as analyte adsorption and is used in combination with a high sieving capacity linear polyacrylamide for efficient fragment separation and resolution (14,16,32). Drawbacks to the use of the Hjerten coating for microchip capillary electrophoresis are the propensity for capillary clogging and difficulties with consistently applying the coating to all capillaries of the microcapillary array. An alternative capillary coating procedure, pDuramide, a dynamic coating polymer, was evaluated for resolution, data quality and ease of use (14, 15). The use of the Long Read linear polymer acrylamide was still necessary as the separation polymer. Measurements for resolution performance, as well as sensitivity tests, both described in the Resolution, Precision and Sensitivity studies, demonstrated that the pDuramide coated chips performed nearly identically to the Hjerten coated chips, with the advantage of greater ease-of-use. Thus, the pDuramide was used exclusively for microchip coating for all subsequent fragment separations.

Resolution and Precision Studies

Measures of resolution and precision were performed in order to assess the performance of the prototype µCAE instrument in combination with Fragment Profiler compared with a commercial capillary electrophoresis instrument employed by many forensic laboratories, the ABI 310. Precision calculations were performed using PowerPlex® 16 allelic ladder samples on both the ABI 310 and the µCAE, using modified Hjerten coated microchips. As shown in Table 1, the ABI 310 displayed precision superior to the µCAE instrument utilized in the VDFS laboratory as well as the µCAE device data obtained from runs performed at the Mathies' laboratory. However, when the performance of the µCAE instrument is compared with reports for commercial multi-capillary array instruments, rather than the single capillary ABI 310, the performances for the ABI 3100, 3700 and the MegaBACE 1000 are essentially equivalent to the µCAE device. The sizing precision for the µCAE device using data produced at the Mathies' laboratory displayed a wider range than that produced at VDFS. That is likely due to the optimization of the instrument run parameters and software applications that were ongoing during the time frame in which the data were generated in the Mathies' laboratory as well as the less rigorous ambient temperature control compared to VDFS. Conditions utilized at VDFS varied little from those reported in the Yeung *et al.* paper.

Resolution measurements were derived as described in the Buel *et al.* report (31), providing a broad evaluation of capillary electrophoresis resolution. Larger molecular weight loci, such as CSF1PO, as well as the smallest molecular weight locus, Amelogenin, were evaluated as described, using PowerPlex® 16 allelic ladder

samples. As seen in Figure 2 and Table 2, the base resolution (R_b), resolution length (RSL) and the valley value (Vv) are similar to those reported by various sources for the ABI 310 and those measured by Yeung *et al* for the µCAE device using the TH01 locus. Values were also calculated by VDFS for the µCAE device using data generated in the Mathies' laboratory using loci (CSF1PO, TPOX and Amelogenin) in addition to TH01. This was performed in order to compare it to the µCAE device operation at VDFS utilizing all of the same loci as Buel *et al.* for direct comparison. In addition, VDFS calculated values from µCAE device runs performed at VDFS using microchips coated with the modified Hjerten procedure as well as the pDuramide dynamic polymer coating. Values produced from data generated at VDFS for microchips coated using the two coatings were nearly identical and were similar to those produced for the µCAE device operated in the Mathies' laboratory. Moreover, peak morphology and the separation between the 9.3 and 10 alleles of the TH01 allelic ladder were virtually identical between the two different microchip coating procedures and were very similar to that produced by the ABI 310 (Figure 2). Furthermore, the pDuramide coated µCAE microchips demonstrated a significantly greater number of open, unclogged capillaries than the modified Hjerten coated microchips; an average of approximately 20% more open capillaries were obtained (n=7 runs using Hjerten coating for a total of 672 capillaries; n=8 runs using pDuramide for a total of 768 capillaries; data not shown).

Sensitivity Assays

Evaluation of instrument sensitivity is essential for validation studies and performance checks. Sensitivity assays were performed for both the modified Hjerten coated chips, using a National Institute of Standards and Technology (NIST) provided sensitivity series and the pDuramide coated chips, using sensitivity series generated from a DNA sample prepared at VDFS. As demonstrated in Table 3 and displayed in Figure 3, the sensitivity for the μCAE device was comparable to that reported for commercial capillary electrophoresis instruments (33,34) and to that reported by Yeung *et al*. Moreover, the coating procedure applied to the microchips did not appear to affect sensitivity.

Mixture Studies

As with sensitivity assays, mixture studies are a critical component to validation work. Table 4 displays the PowerPlex® 16 typing results for a mixture study. While the majority of minor contributor alleles were observed at the 3:1 and 1:3 ratios, at the 3:1 ratio, a 12 allele at D13S317 was below the threshold for reporting as were a 9 allele at TH01 and an 11 allele at Penta E at the 1:3 ratio. These findings are not unexpected and are consistent with previously reported mixture results using commercial detection platforms (34,35). All minor contributor alleles were reported in the Yeung *et al.* paper for the 1:3 and 3:1 mixture ratios analyzed with the μCAE device. This minor performance difference may be due to the different methods employed for estimating DNA concentration since the mixture samples in the Yeung *et al* paper were prepared by NIST using a different methodology for DNA quantitation than that employed at VDFS (13). Collins *et al.*

(35) reported full detection of the minor contributor at the 3:1 and 1:3 ratios however, the threshold setting was lower than that routinely applied to casework samples (50 rfu) and minor contributor alleles at the stutter position were excluded from analysis, which was not the case in the study reported here. Minor contributor alleles were above the peak threshold at many loci for both the 9:1 and 1:9 ratios as is also consistent with other reports (13,34-36). The dynamic coating polymer, pDuramide, was utilized for microchip coating in this experiment, which again indicated that this alternative coating procedure did not adversely impact µCAE device performance.

Non-probative sample analysis

Nineteen non-probative case samples from five different cases were analyzed using the µCAE device. Non-probative cases included two sexual assaults, a hit-and-run, aggravated battery, and aggravated robbery/aggravated battery. PowerPlex® 16 profiles produced employing the µCAE device were consistent with profiles produced with the ABI 310 using the same PowerPlex® 16 amplicons (data not shown). Although original DNA typing was conducted on the 19 samples using various forensic PCR-based human identification kits, results were in 100% concordance with regards to conclusions that may be drawn from the µCAE device data compared to the original case reported conclusions (C. Crouse, personal observations; Figure 4). As shown in Figure 4, the minor contributor alleles from the sperm DNA which carried over into the non-sperm fraction are clearly visible and the major profile is consistent with the victim profile. This is consistent with the Yeung *et*

al. report which demonstrated that an array of casework could be successfully typed using the µCAE device.

STR Typing using the PowerPlex® YSystem

Mock sexual assault samples, consisting of 6 sperm fractions (1,000 sperm on swab [1K], 10,000 sperm on swab [10K] and 50,000 sperm on swab [50K]) and the 6 corresponding non-sperm fractions, were amplified for PowerPlex® Y and subjected to analysis using both the µCAE device and the ABI 310. Results were concordant between the two instruments and software platforms. However, for some samples with low signal (peak heights close to the 100 rfu threshold on the ABI 310), some peaks could not be labeled if the S/N ratio of 3:1 was applied to data generated using the µCAE device (data not shown). Although no attempts were made to optimize PowerPlex® Y typing of samples using the µCAE device, the majority of the mock sexual assault samples provided STR profiles of similar high quality to those produced by the ABI 310 (Figure 5).

DISCUSSION

Much of the work reported here involved the reproduction of experiments performed in the Mathies' laboratory at the University of California, Berkeley and reported in Yeung *et al.* (2006). While Yeung *et al.* clearly demonstrated that the µCAE device performance was consistent with industrial capillary electrophoresis instruments, all without the benefit of commercial, customized software, further demonstration of instrument utility in a forensic laboratory was merited. We report

the successful transfer of this technology from an academic environment to a forensic laboratory, clearly demonstrating the feasibility of using a microfabricated capillary electrophoresis system, capable of both rapid and high throughput STR typing for forensic DNA profiling..

The precision study was performed solely using the modified Hjerten coated microchips and displayed a sizing precision comparable to commercially available multi-capillary array instruments. This result is of import as neither the software nor the separation polymer have been commercially optimized or customized for use with the µCAE device.

The resolution data produced by the µCAE device was very similar to data produced by the ABI 310 and reported by various laboratories; data quality appeared to be unchanged when compared with data generated using the modified Hjerten procedure. The study comparing the resolving power of microcapillary array chips coated using the modified Hjerten coating with those coated using p-Duramide, showed no statistical difference in performance between the two coating procedures. Given the greater ease of application and shorter preparation time, pDuramide became the coating of choice. .

The sensitivity assays produced results similar to those observed with commercial systems as well as results reported for the µCAE system by Yeung *et al*. The apparent sensitivity was also consistent between the two different microchip coating methods for two different sensitivity series; one prepared internally and one by NIST. The conversion from the modified Hjerten coating procedure to the dynamic coating polymer, pDuramide, proved to be expeditious and fortuitous. Not

only was the resolution comparable to that obtained using the modified Hjerten procedure, but a reproducibly greater proportion of the capillaries in the 96 capillary microarray remained open and useful for genotyping, thereby circumventing a predominant obstacle for the efficient performance of microchip capillary array electrophoresis. An added bonus was that the pDuramide coating process required less time (~30 minutes versus ~2 hours) and was far easier to apply.

The successful PowerPlex® 16 STR profiling of 19 non-probative casework samples using the μCAE device demonstrated that the system installed at VDFS was correctly operated and was capable of rapidly and accurately typing the samples. These findings are also consistent with the previously reported data generated by Yeung *et al*.

Finally, successful typing of PowerPlex® Y amplified mock casework samples using the μCAE device further demonstrated its functionality and attests to ease of use of the final instrument and protocol. While the tested instrument is not a commercially manufactured system and lacks the rigor of production line assembly and a customized software, the μCAE device still provides sufficient ease of use that one can run different STR typing systems without changing the operation parameters.

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Table 1. Sizing Precision

Instruments	Sizing precision (S.D.) Within-run	Between-run	± 3 S.D.
ABI 310 (VDFS)	0.03-0.06 bp	0.03-0.06 bp	± 0.18 bp
µCAE (VDFS)	0.02-0.23 bp	0.08-0.14 bp	± 0.42 bp
µCAE (Mathies' lab data)	---	0.11-0.31 bp	± 0.93 bp
ABI 377[37]	0.01-0.09 bp	---	---
ABI 377[38]	0.03-0.10 bp	---	---
ABI 310[30]	---	0.02-0.12 bp	± 0.36 bp
ABI 310[37]	---	0.04-0.12 bp	---
ABI 310[39]	0.10 bp	0.20 bp	---
ABI 310[40]	0.01-0.13 bp	≤0.16 bp	---
ABI 3100[38]	---	0.03-0.17 bp	---
ABI 3700[38]	---	0.02-0.21 bp	---
FMBIO II[35]	---	---	± 0.40-0.80 bp
MegaBACE 1000[38]	---	0.04-0.17 bp	---

Table 2. Measurements of Resolution

Instruments	Rb	RSL	Vv
ABI 310 (VDFS)	1.15-1.72	0.67-0.99	0.64
µCAE (Mathies' lab data)	1.30-1.61	0.74-1.04	0.73
µCAE (VDFS) Hjerten coating	1.35-1.53	0.78-0.91	0.80
µCAE (VDFS) pDuramide coating	1.31-1.54	0.78-0.904	0.74
ABI 310[31]	1.04-1.64	0.61-0.96	0.51
ABI 310[40]	1.24-1.31	---	0.43-0.49
ABI 310[37]	---	---	~0.30
ABI 310[30]	1.13-1.49	---	---
µCAE[13]	1.3	0.76	---

Table 3. Sensitivity Data.

VDFS DNA sample – pDuramide coating

In house sample	FGA	TPOX	D8	vWA	Amel	P.E.	D18	D21	TH01	D3	P.D.	CSF	D16	D7	D13	D5
2 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
1 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
0.5 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
0.25 ng	22,24	8,11	10,13	18,20	XY	5,13	11,15	29,30	9	15	10,11	10,11	10,11	11	10,11	11,13
0.125 ng	22,24	8,11	10,13	18,20	X,Y	5,13	11,15	29,30	9	15	10,11	10	10,11	11	10,11	11,13
0.062 ng	-	-	-	-	X,Y	-	11	-	9	15	-	-	-	-	-	
0.031 ng	-	8,11	-	18	X,Y	5	15	-	9	15	-	-	-	-	-	-
0.015 ng	-	-	-	-	Y	-	-	-	9	15	-	-	-	-	-	-

NIST sample – modified Hjerten coating

NIST sample	FGA	TPOX	D8	vWA	Amel	P.E.	D18	D21	TH01	D3	P.D.	CSF	D16	D7	D13	D5
10 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
5 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
2.5 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
1.25 ng*	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
0.62 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
0.31 ng	19,23	8	14	17,18	X,Y	5,16	14,16	31.2,33.2	6,7	16	11,14	11,12	9,11	9,11	8,12	11,12
0.15 ng	19	8	14	17,18	X,Y	5	14,16	31.2,33.2	7	16	14	12	9	11	-	-

*Data for sample obtained from a µCAE device run performed on a different day.

Table 4. Mixture study

Mixture Data Sample #1:Sample #2

	1:0	9:1	3:1	3:2	2:3	1:3	1:9	0:1
Sample 1	**14/14**	**14/14**	**14/14**	**14/14**	**14/14**	**14/12***	**8/14**	**0/14**
Sample 2	**0/14**	**4/14**	**13/14**	**14/14**	**14/14**	**14/14**	**14/14**	**14/14**

KEY: 16/16 indicates all of the PowerPlex 16 loci amplified and were correctly typed. All numbers less than 16 indicate the number of loci that were successfully typed.
Note: CSF1PO and Amelogenin loci excluded since alleles were identical for both samples.
* At two of the loci, only one of the minor contributor alleles was observed above peak threshold.

Figure 1

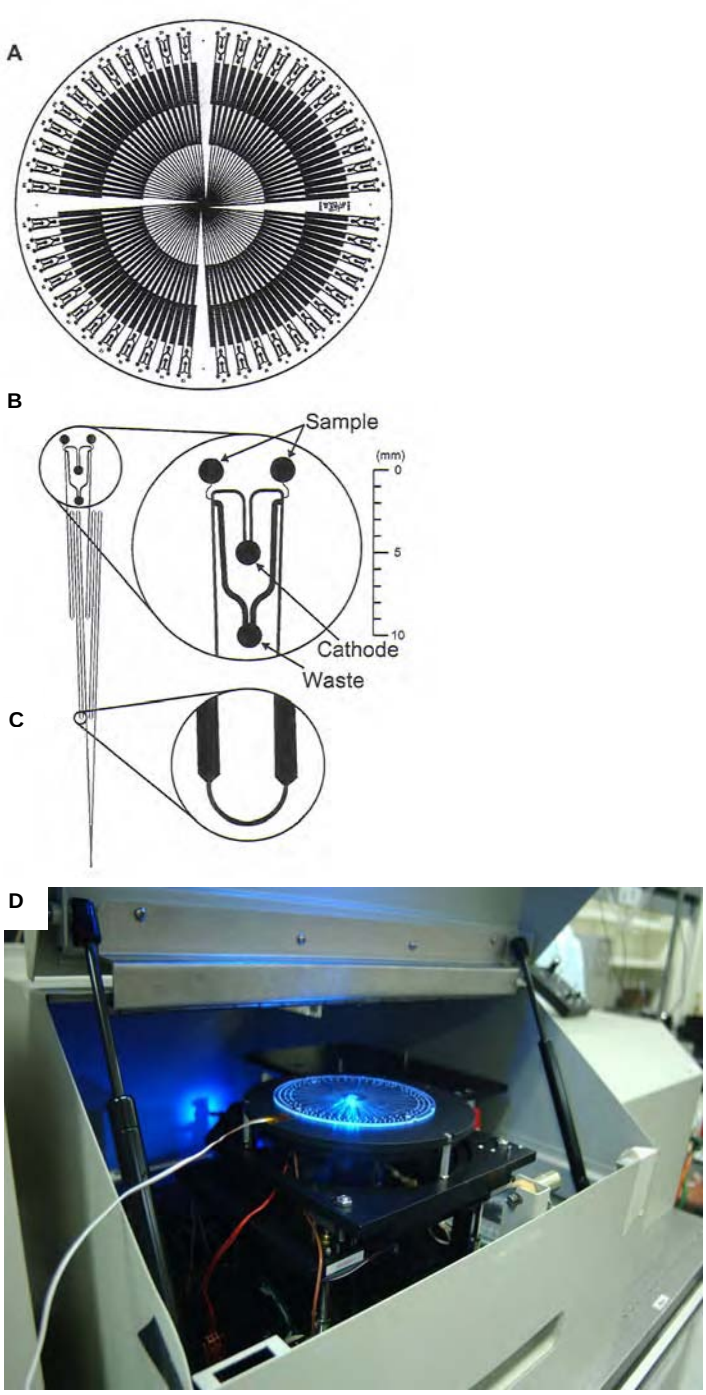


Figure 2

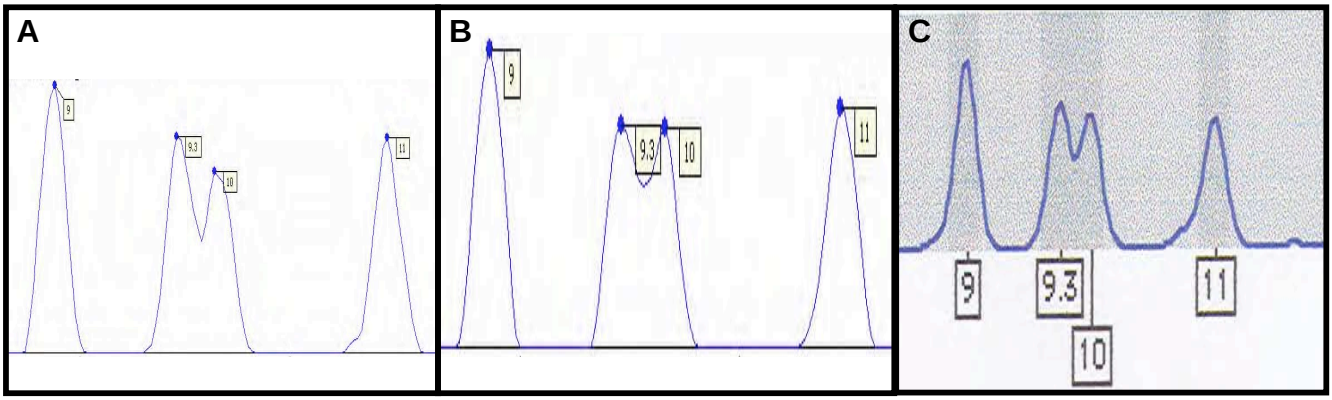
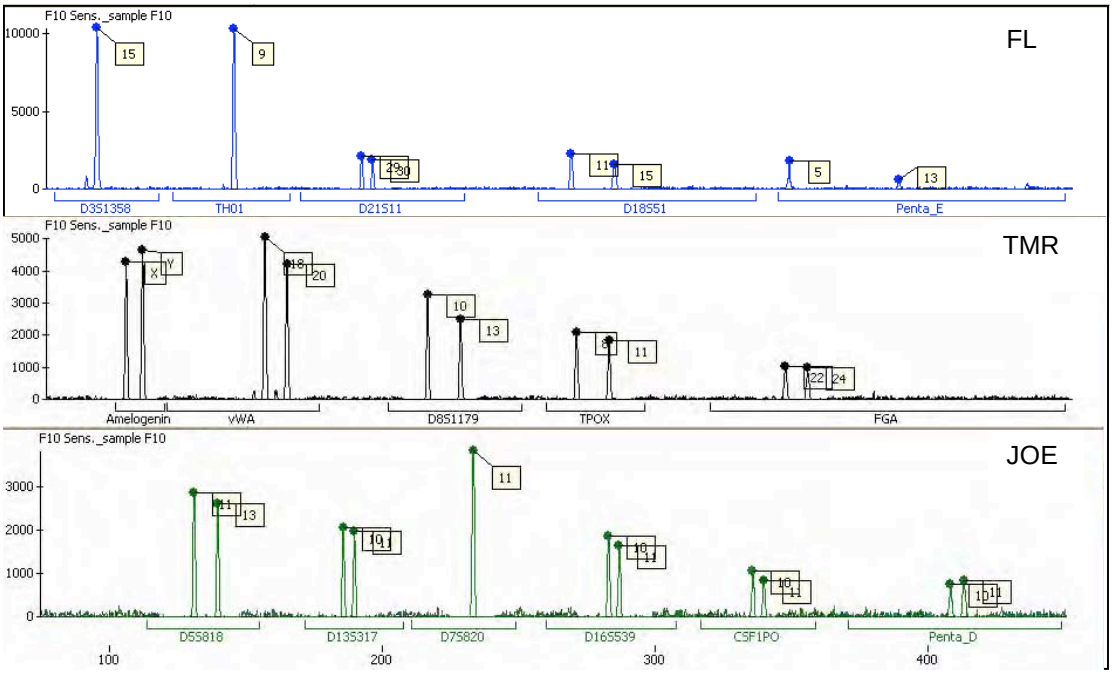


Figure 3

A.



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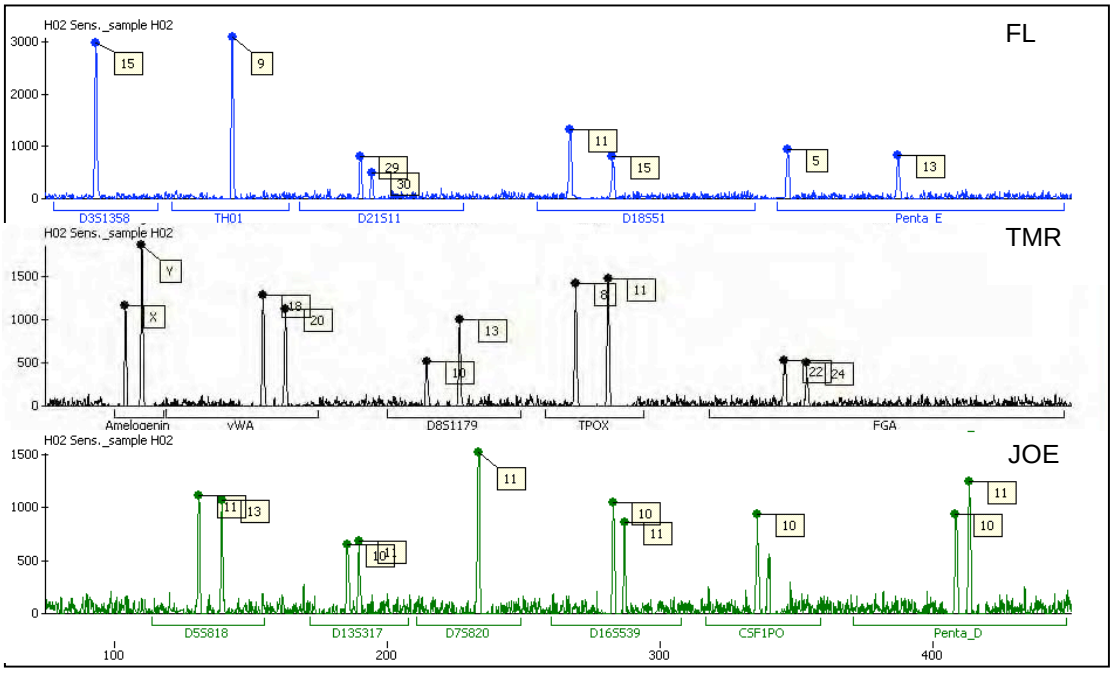
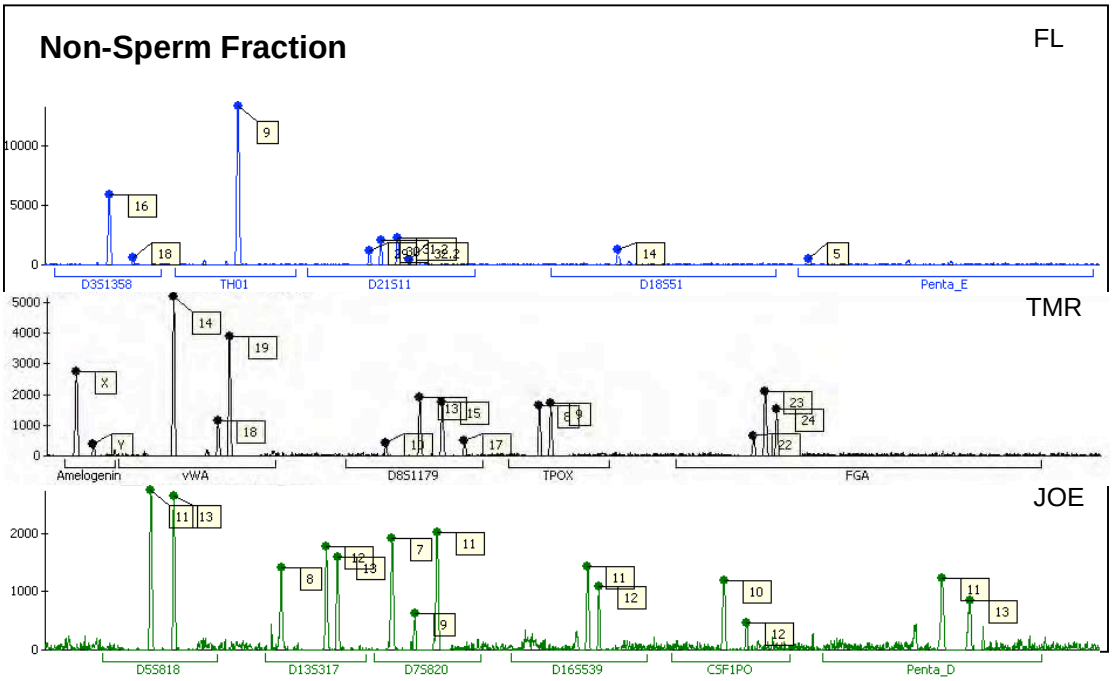


Figure 4.



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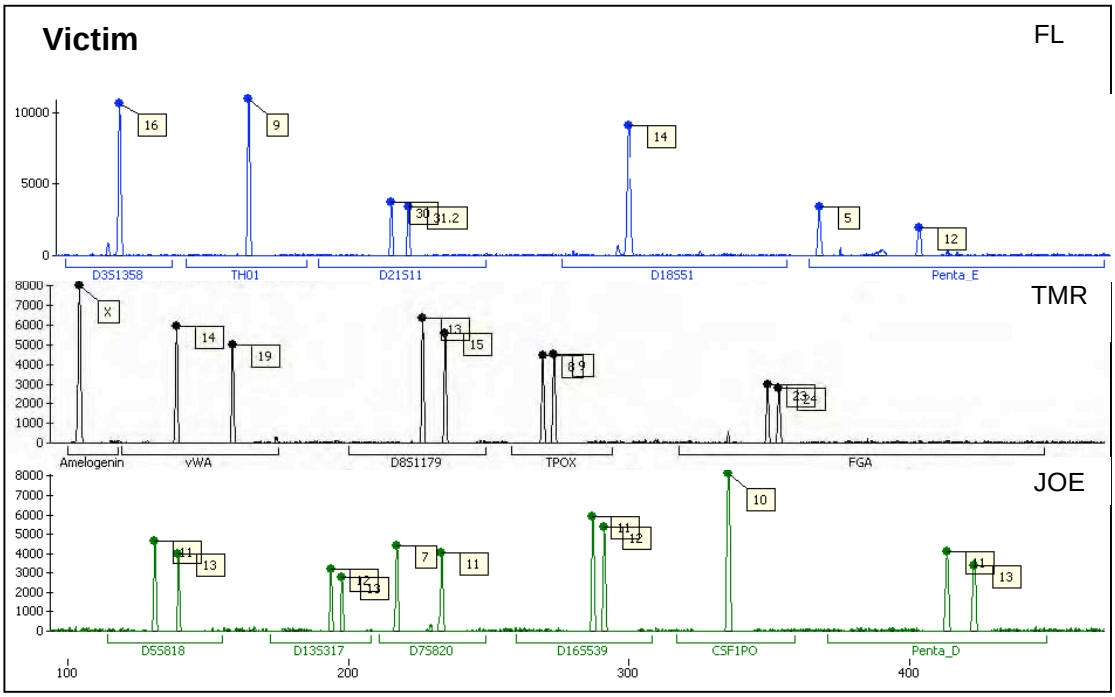

Figure 5.

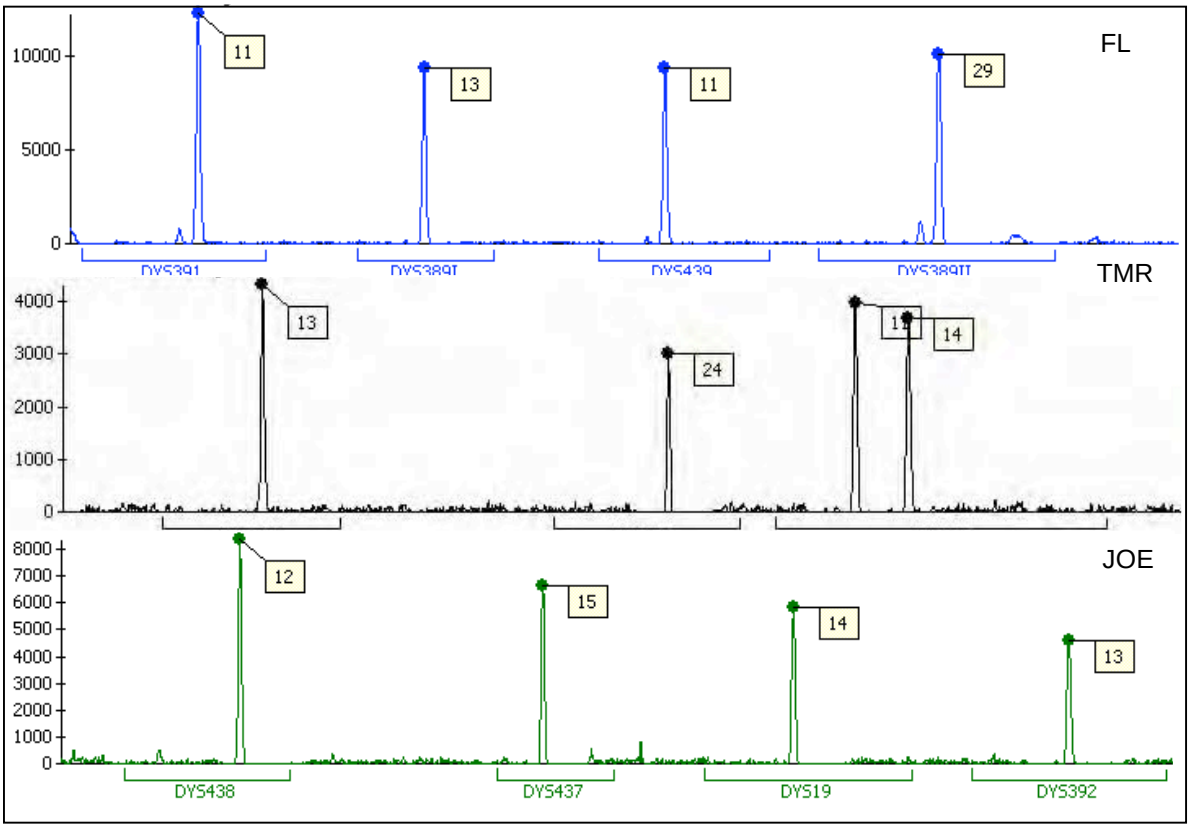

FIGURE LEGENDS

Figure 1. (A) Design of the 96-channel microfabricated capillary array electrophoresis (µCAE) microchip used at VDFS. (B) Expanded view of channel doublet including two sample reservoirs and the common cathode and waste reservoirs. (C) Expanded view of a hyperturn within the channel. (D) Photograph of uCAE device prototype installed at VDFS. Figure modified from Paegel *et al*.(9).

Figure 2. Separation of the 9.3/10 alleles of PowerPlex® 16 allelic ladder obtained on, (A) µCAE chip using the pDuramide, dynamic polymer coating. (B) µCAE chip using the modified Hjerten coating. (C) ABI 310 Genetic Analyzer.

Figure 3. PowerPlex® 16 profiles of sensitivity study samples using a single source male DNA amplified with, (A) 2 ng of input DNA and, (B) 125 pg of input DNA.

Figure 4. Non-probative sample analysis by the µCAE device using PowerPlex® 16. (A) Non-sperm fraction mixed profile. (B) Victim profile.

Figure 5. PowerPlex® Y profile generated from the sperm fraction of the 50K buccal, mock sexual assault swab.

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Integrated Portable Polymerase Chain Reaction-Capillary Electrophoresis Microsystem for Rapid Forensic Short Tandem Repeat Typing

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A portable forensic genetic analysis system consisting of a microfluidic device for amplification and separation of short tandem repeat (STR) fragments as well as an instrument for chip operation and four-color fluorescence detection has been developed. The microdevice performs polymerase chain reaction (PCR) in a 160-nL chamber and capillary electrophoresis (CE) in a 7-cm-long separation channel. The instrumental design integrates PCR thermal cycling, electrophoretic separation, pneumatic valve fluidic control, and four-color laser excited fluorescence detection. A quadruplex Y-chromosome STR typing system consisting of amelogenin and three Y STR loci (DYS390, DYS393, DYS439) was developed and used for validation studies. The multiplex amplification of these 4 loci with 35 PCR cycles followed by CE separation and 4-color fluorescence detection was completed in 1.5 h. All the amplicons can be detected with a limit of detection of 20 copies of male standard DNA in the reactor. Real-world forensic analyses of oral swab and human bone extracts from case evidence were also successfully performed. Mixture analysis demonstrated that a balanced profile can be obtained even at a male-to-female template ratio of 1:10. The successful development and operation of this portable PCR−CE system establishes the feasibility of rapid point-of-analysis DNA typing of forensic casework, of mass disaster samples or of individuals at a security checkpoint.

Short tandem repeat (STR) assays have become an indispensable and routine technique in modern forensic casework since their first application in 1991.[1] Polymerase chain reaction (PCR)-based amplification of multiple STR loci followed by capillary electrophoretic (CE) separation provides STR assays with high sensitivity and high discrimination power.[2−4] In addition to forensic identification, STR assays have found application in paternity testing, missing person investigations, human identification in mass disasters, evolution, and clinical diagnosis.[5,6] However, the limited capabilities of current genotyping technologies, which are time-consuming, labor-intensive, and expensive, have resulted in backlogs in forensic laboratories around the world. To address these issues, high-throughput and integrated instruments are needed to improve the data productivity. In addition, rapid and portable DNA typing devices that can provide on-site forensic analysis could be valuable in crime scene investigation and for law enforcement and security applications.

In the quest to produce portable, real-time analytical devices as well as high-throughput analyzers, microfabricated microfluidic analysis systems, so-called micro total analysis systems (μTAS), have attracted increasing attention due to their ability to integrate multiple molecular biology processes at the microliter to nanoliter scale in a single device. Since the inception of μTAS in 1990,[7] much progress has been made to miniaturize and integrate DNA analysis steps into a microchip format,[8,9] including DNA extraction,[10−12] PCR amplification,[13,14] and CE separation.[15−17] These technologies are now beginning to be translated to forensic applications.

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In 1997, Ehrlich's group demonstrated that a quadruplex STR system (*CSF1PO, TPOX, THO1, vWA*) could be separated with high accuracy in less than 2 min by microchip capillary electrophoresis.[18] More recent work in our group demonstrated the use of a 96-channel microfabricated capillary array electrophoresis device coupled to a four-color confocal fluorescence scanner for high-performance STR typing using both the PowerPlex 16 and AmpF*l*STR Profiler Plus multiplex PCR systems.[19] The separations were completed in less than 30 min with single-base resolution on 96 CE channels simultaneously. Although these systems heavily rely upon conventional off-chip sample preparation, they do indicate that chip-based CE technology is poised for application in forensic laboratories.

The on-chip integration of DNA sample amplification by PCR has also been demonstrated. An integrated PCR−CE microdevice consisting of a silicon reaction chamber attached to a glass CE analysis chip was developed in our laboratory in 1996 to amplify and analyze PCR products, providing rapid reaction times, low sample consumption, and potential on-chip integration with other analytical techniques.[20] Since then, great progress has been made in the development of PCR microdevices, including alternative chip formats (flow-through and stationary chamber), substrate materials (silicon, glass, and polymer), and heating methods (contact and noncontact heating).[21,22] However, most of these systems either require a high starting template concentration or are not suitable for integration with CE separation.

Based on the development of integrated PCR−CE microdevices by Lagally et al.,[23−25] a fully integrated portable PCR−CE microsystem was recently demonstrated for pathogen detection applications. The limit of detection for this system was 2−3 *Escherichia coli* cells, and the amplifications required only 20 min.[26] More recently, a nanoliter-scale microdevice was developed, that integrates the three Sanger sequencing steps: thermal cycling, sample purification, and capillary electrophoresis.[27] Building on this work, a four-lane integrated PCR−CE array microdevice was also demonstrated to amplify femtogram amounts of DNA followed by electrophoretic separation in less than 30 min.[28] These advances raise the possibility that these technologies can also be used for forensics whose stringent requirements include high efficiency and balanced amplification of multiple STR loci, reproducible electrophoretic separation under denaturing conditions, and high-sensitivity, four-color fluorescence detection.

Here we present the design and operation of a new PCR−CE microdevice for forensic STR analysis, as well as a new portable analysis instrument, which contains all the electronics and optics for temperature control, microfluidic manipulation, CE separation, and four-color fluorescence detection. To explore the utility of this system for forensic DNA typing, a quadruplex STR system was developed with amelogenin, a sex-typing marker, and three Y chromosome STR loci. As over 89% of violent offenses are committed by men,[29] Y-STR assays have a unique value in forensic DNA typing, particularly in sexual assault cases.[6,30,31] Due to the lack of recombination, Y-STR assays have also become a popular tool for paternity testing, evolutionary studies, and historical and genealogical research.[6] With this quadruplex Y-STR system, we evaluated the limit of detection of the portable PCR−CE microsystem as well as its ability to analyze forensic casework samples and to detect male DNA in a background of female DNA.

EXPERIMENTAL SECTION

Microdevice Design. The microdevice contains two identical PCR−CE systems, symmetrically arranged on the 4-in. wafer (Figure 1A). The structure of each system is similar to the device developed in our group previously,[26] but the design has been adapted for the portable instrument. Each system consists of a 160-nL PCR chamber, an integrated heater, a four-point resistance temperature detector (RTD), two poly(dimethysiloxane) (PDMS) microvalves, and a 7-cm-long CE separation channel. The PCR reactor region with the relative positions of the PCR chamber, heater, and RTD is shown in Figure 1B.

The microdevice is composed of a glass manifold, a PDMS membrane, a glass heater/channel wafer, and a glass RTD wafer (Figure 1C). The PCR chamber (bottom side of the heater/channel wafer) and the RTD (top side of the RTD wafer) are positioned next to each other after bonding. The microfabricated PCR heater is deposited on the top side of the heater/channel wafer and covers the PCR chamber and the RTD to facilitate thermal cycling under the control of the temperature feedback from the RTD. The PCR chamber contains three exits, two of which are connected to a loading reservoir and a vent reservoir, respectively, through microvalves for the sample loading. The last exit is coupled to a CE separation channel through a narrow injection channel. The glass manifold wafer actuates the PDMS microvalves for fluidic control.[32]

PCR Heater Design. The design of the microfabricated PCR heater is intended to create uniform heating over the entire PCR chamber and to facilitate fast thermal response times. In general, the edges of the heater show the most deviation from temperature set point due to the higher thermal dissipation. To adequately maintain the entire chamber volume at a single temperature and keep the thermal mass of the PCR system as low as possible, the thermal power at the extremities of the heater was increased to diminish the temperature deviation. The PCR heater was designed in an iterative process using computational simulation as a guide.

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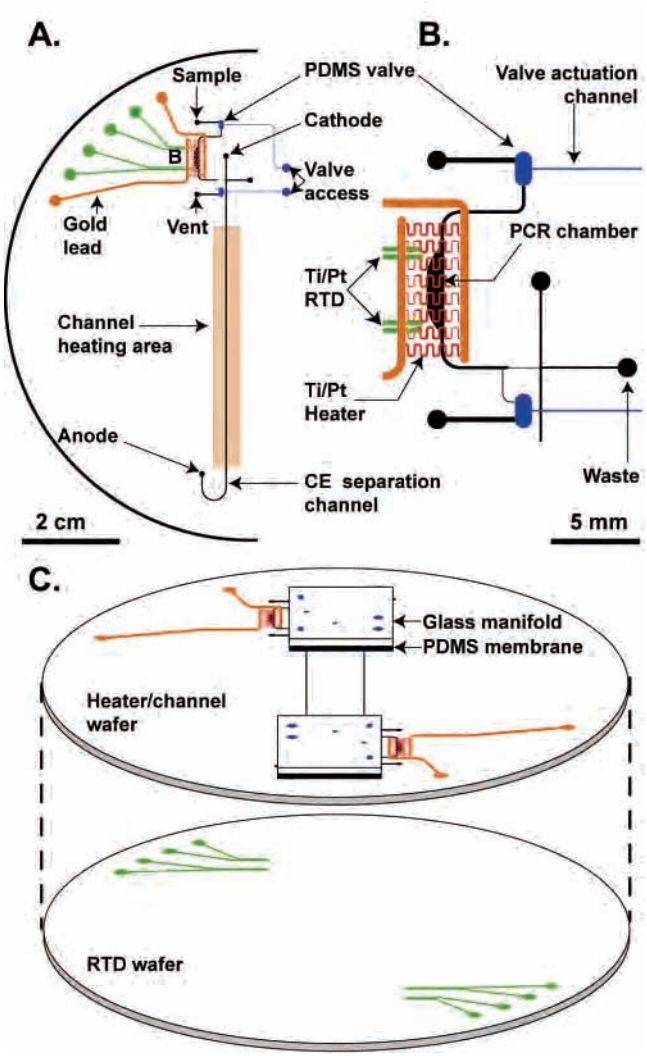

Figure 1. (A) Mask design for the PCR−CE microchip. The glass microchannels are indicated in black, the microfabricated RTD and electrodes are in green, the heater is shown in red, the gold leads of the heater are in gold, and the valves are drawn in blue. (B) Expanded view of the heater, RTD, PCR chamber, and CE injector. (C) Exploded view of the assembly of the PCR−CE microchip, showing the RTDs on the upper surface of the RTD wafer, as well as the glass microchannels etched in the lower surface and the heaters fabricated on the upper surface of the heater/channel wafer.

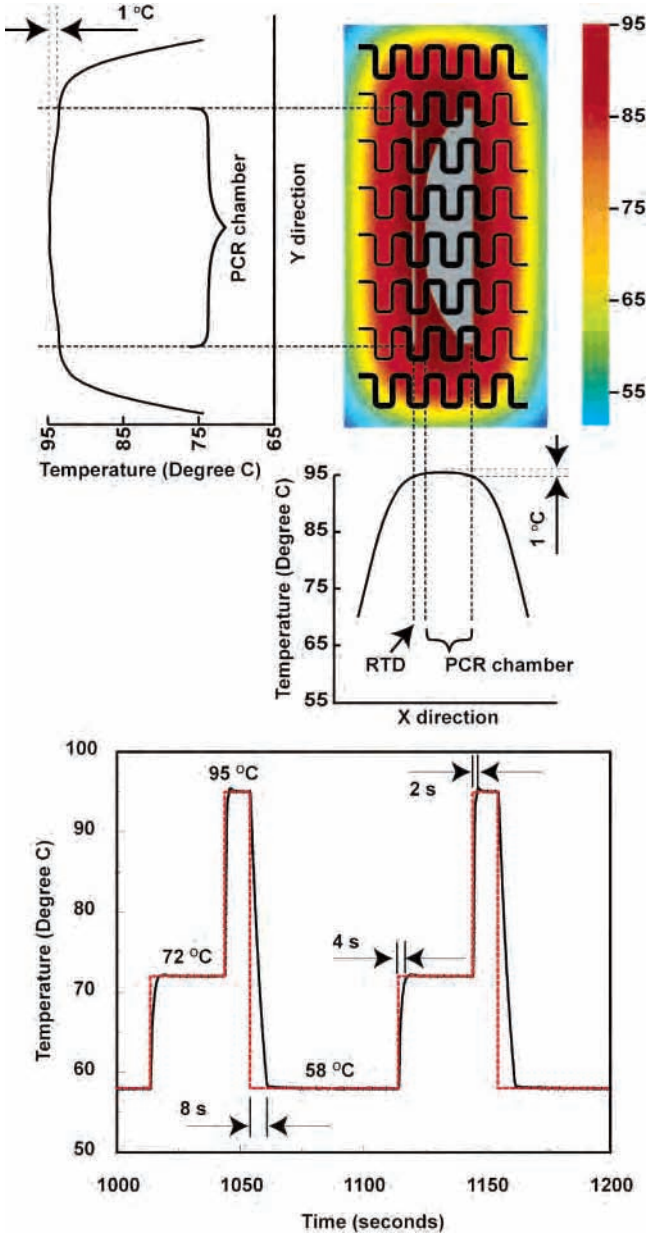

Figure 2. (Top) Color contour plot of the simulated temperature distribution of the PCR chamber layer at 95 °C. By varying the widths of the heating elements in the different regions of the heater, a uniform profile was achieved. The differences between the center and the edge of the heater are only 1 °C. (Bottom) Thermal cycling amplification profile. Black line shows the measured temperature from the RTD and red line is the set temperature. Temperature ramp rates were 11.5 °C /s for heating and 4.7 °C /s for cooling.

As shown in Figure 2, an optimized heater design contains eight serpentine heating elements connected to gold leads in parallel. The width of each heating element in the center region was set to 140 μm. Optimal heating distribution was achieved by narrowing the width to 70 μm on the ends of the central six heating elements, and to 130 μm on the outer two heating elements. Figure 2 (top) shows a color contour plot of the simulated temperature distribution of the PCR chamber at 95 °C using FEMLAB 2.3 (COMSOL, Inc., Burlington, MA). Using this design method, the temperature differences between the center and the edge of the PCR chamber were reduced to less than 1 °C in both the *X* and *Y* directions. Figure 2 (bottom) presents two typical PCR cycles. The temperature ramp rates can reach 11.5 °C/s for heating and 4.7 °C/s for cooling without any active cooling.

Microfabrication. The microfabrication process is similar to that described previously.[25,26] Briefly, to form the heater/channel wafer, a 550-μm-thick D263 glass wafer was coated with 2000-Å amorphous silicon on one side and 200-Å Ti and 2000-Å Pt on the other side. The channel pattern was photolithographically transferred to the amorphous silicon side, and then the sacrificial silicon was etched using SF_6 in a parallel-plate reactive ion etching system creating a hard mask for subsequent glass etching. The exposed glass was etched to a depth of 38 μm in a 49% hydrofluoric acid bath. After etching, the photoresist and silicon were removed using acetone and SF_6, respectively. The integrated PCR heaters were fabricated on the Ti−Pt side of the same wafer. Using a backside contact aligner, a pattern defining the gold heater leads was photolithographically transferred to the surface. Gold was

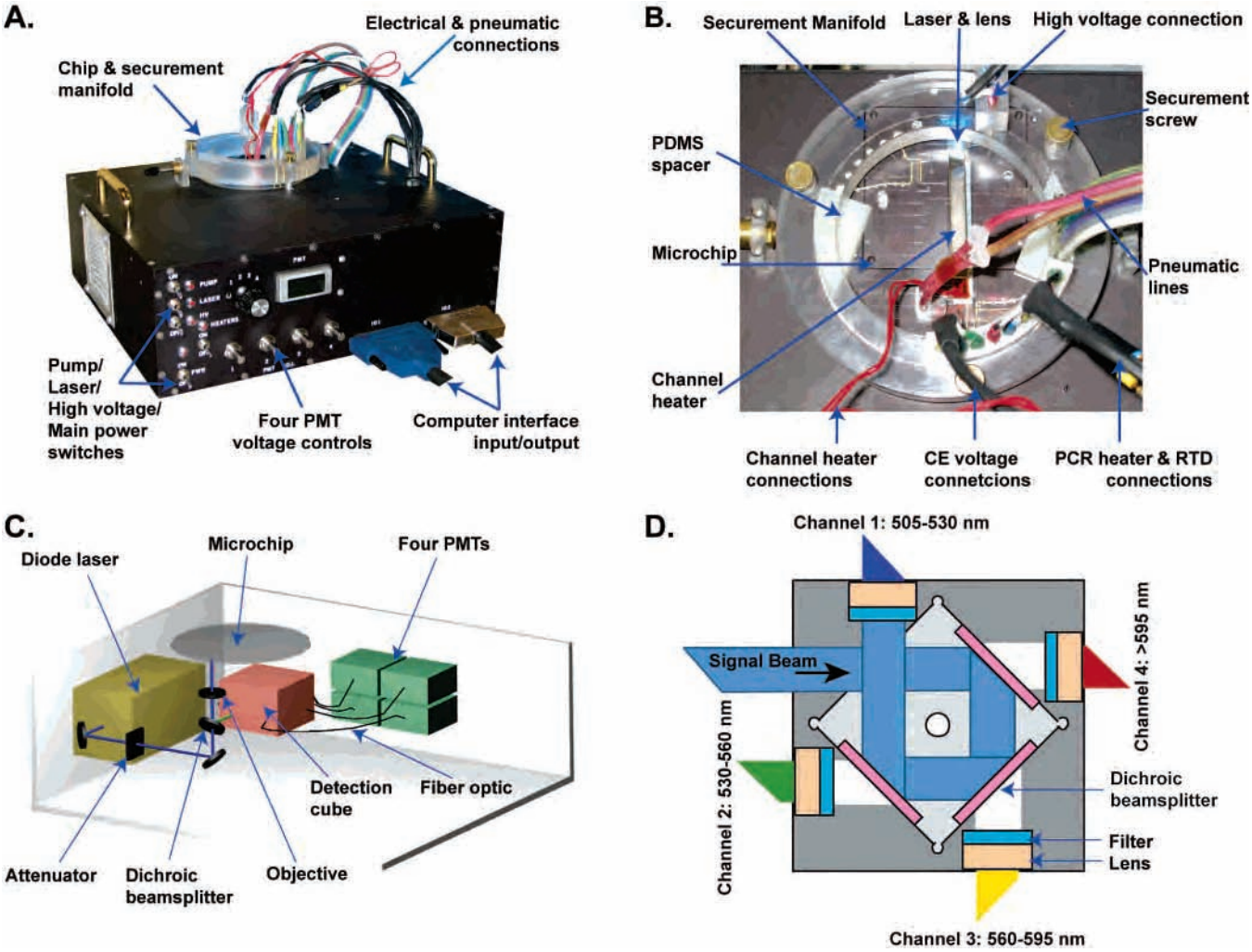

Figure 3. (A) Photograph of the portable PCR−CE system. The analysis system box has dimensions 12 × 10 × 4 in. (B) Closeup of the microchip and the manifold. A plexiglass manifold was used to fix the microchip in place and supply the electrical and pneumatic connnections to the chip. (C) The schematic of confocal fluorescence detection system. (D) Expanded top view of the four-color detection cube.

electroplated onto the open Ti−Pt seed layer to a thickness of 5 μm to form the heater leads. Photoresist was then removed, and the wafer was repatterned to define the heating elements. Using an ion beam etching system, the heating elements were etched into the Ti−Pt seed layer. Finally, holes were drilled using a CNC mill for via holes, fluidic reservoirs, and electrical and pneumatic access holes.

To form the RTD wafer, a 700-μm D263 glass wafer coated with 200-Å Ti and 2000-Å Pt was patterned with photoresist and etched using hot aqua regia. To form the glass microchannels and PCR chambers, the RTD wafer and the heater/channel wafer were thermally bonded in a vacuum furnace at 580 °C for 6 h. The glass manifold was fabricated from a 700-μm D263 glass wafer using the same glass etching method described above and diced into 23 × 18 mm pieces. The microvalves were assembled by cleaning the PDMS membrane in a UV−ozone cleaner for 1 min and then sandwiching the membrane between the bonded wafer stack and the glass manifold. This method results in a tight but reversible glass−PDMS bonding.

Instrumentation. The instrument used to perform analyses with the microdevice is shown in Figure 3A and B. The instrument contains a 488-nm, frequency-doubled diode laser, an optical system for detecting four different fluorescence signals, pneumatics for the on-chip PDMS microvalves, electronics for PCR temperature control, and four high-voltage power supplies for CE. The weight of the instrument is 10 kg with a power consumption of 20 W, which can be supplied by a car battery. A LabVIEW graphical interface (National Instruments, Austin, TX) developed in-house was used to control the system through two DAQ boards (National Instruments).

The schematic of the detection system is shown in Figure 3C. The beam from the laser (Protera, Novalux Corp., Sunnyvale, CA) is reflected by a dichroic mirror (505DCXT, Chroma Technology Corp., Brattleborro, VT) into an attenuator that limits the power intensity of the laser beam to 4 mW (measured from the objective). Then, the attenuated beam is reflected by a second dichroic mirror (505DCXT), passes through a dichroic beam splitter (488DCSXBP, Chroma), and is focused into the channel in the microdevice with a custom-built objective (0.70-mm focal length in D263 glass, 0.88 NA). The returning fluorescent signal is collected by the objective and reflected into a four-color confocal detection cube by the dichroic beam splitter. As shown in Figure 2D, the detection cube separates fluorescent light into four distinct channels, blue (505−530 nm), green (530−560 nm), yellow (560−595 nm), and red

Table 1. Locus Information, Dye Labeling, and Primer Sequences

locus	repeat motif	dye label[a] and primer sequence (5' → 3')	9948 male standard DNA	
			repeat number	amplicon size (bp)
amelogenin		Fwd: [FAM-FAM]-CCCTGGGCTCTGTAAAGAA Rev: ATCAGAGCTTAAACTGGGAAGCTG	X, Y	106, 112
DYS390	[TCTG]-[TCTA][c]	Fwd: [FAM-R6G]-CTGCATTTTGGTACCCCATA Rev: GCAATGTGTATACTCAGAAACAAGG	24	171
DYS393	[AGAT]	Fwd: [FAM-TMR]-AACTCAAGTCCAAAAAATGAGG Rev: GTGGTCTTCTACTTGTGTCAATAC	13	123
DYS439	[GATA]	Fwd: [FAM-ROX]-ACATAGGTGGAGACAGATAGATGAT Rev: **G**CCTCAAGTGATCCACCCAAC[b]	12	191

[a] FAM, 5-carboxyfluorescein; R6G, rhodamine 6G; TMR, *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-x-rhodamine. [b] The 5′ ***G*** of the DYS439 reverse primer was added to promote adenylation. [c] Compound tetranucleotide repeat.

(>595 nm), by sequential reflection from a serial of dichroic beam splitters (595DCXR, 570DCXR, 537DCLP, Chroma). Fluorescent light is further filtered by a filter in each channel (Ch1, D520/26 m; Ch2, D550/20 m; Ch3, D580/26 m; Ch4, E600LP, Chroma). The filtered light is focused by an achromat lens (45208, Edmund Optics, Barrington, NJ) into an optical fiber (Newport Corp., Irvine, CA), the entry of which functions as a confocal pinhole and is provided with xyz adjustment, and then guided by an optical fiber to the desired PMT (H9306-03, Hamamatsu Corp., Bridgewater, NJ). The four signals are processed using an active 5-Hz, low-pass filter and collected at 10 Hz using the 16-bit DAQ board.

The microdevice is placed onto a recessed area on the top of the instrument and held in place with a plexiglass manifold. Two PDMS spacers are used to support the manifold and provide a soft contact to the microdevice. The manifold contains six spring-loaded pins pressed against the electrical pads on the device, providing the connections for sensing the RTD and powering the PCR heater. The manifold also contains Pt electrodes that are positioned within the reservoirs on the microchip for application of high voltages during CE. A thin-film heater, (9.2 Ω, Minco, Minneapolis, MN), sandwiched between the microchip and a magnet, is used to heat the CE separation channel. Flush contact between the magnetic heater and the chip is obtained by embedding a steel bar on the surface of the instrument.

The design of the electrical circuits for driving the RTD and heater is the same as presented eariler.[26] Briefly, a 4-mA current source powers the RTD through the outer set of leads, and the resulting voltage is sensed through the inner set. The signal is processed using an active low-pass filter at 5 Hz and then transferred to the DAQ board. Temperature control is accomplished through a proportion/integrator/differentiator module within the LabVIEW program, which outputs through the DAQ board to control the PCR heater power supply within the instrument.

The PDMS microvalves are controlled using vacuum or pressure supplied through pneumatic connections to the valve access holes on the glass manifold. Eight pneumatic lines are available for fluidic control. Each line can be switched between vacuum (open valve) and pressure (closed valve) using a solenoid valve (H010E1, Humphrey, Kalamazoo, MI) controlled through the DAQ board. Pressure (4.5 psi) and vacuum (−8 psi) were separately supplied by two rotary pumps (G12/02−8-LC, Thomas, Sheboygan, WI) inside the instrument.

Microdevice Preparation. Before operation, the channels were first coated for 1 min with a dynamic coating diluted 1:1 with methanol (DEH-100, The Gel Co., San Francisco, CA) to minimize electroosmotic flow. The separation matrix, 5% (w/v) linear polyacrylamide with 6 M urea in 1× Tris TAPS EDTA (TTE) buffer, was loaded from anode reservoir with a syringe to fill the entire CE separation system. A prepared PCR mixture (10 μL) was pipetted into the sample reservoir. Vacuum applied at the vent reservoir moved the sample into the PCR chamber, and a gel−solution interface was formed at the end of the narrow injection channel. This interface functioned as a passive barrier to prevent the flow of reagents into the CE channels during thermal cycling. Using this method bubble-free loading of the PCR reactor was consistently achieved. After sample loading, the PDMS microvalves were closed by applying pressure to prevent hydrodynamic flow.

PCR Amplification and Capillary Electrophoresis. PCR amplifications were conducted from 9948 male and 9947A female genomic DNA (Promega commercial genomic DNA controls, Promega, Madison, WI), as well as two samples from forensic casework previously processed by the Palm Beach County Sheriff's Office. These casework samples were extracted from an oral swab and human bone, respectively, using the DNA IQ system (Promega), and then quantified using Quantiblot (Applied Biosystems, Foster City, CA) with Hitachi CCDBio (Hitachi, Alameda, CA) signal detection. All the DNA templates were also amplified in a traditional thermal cycler and analyzed in an ABI Prism 3100 genetic analyzer (Applied Biosystems) to obtain the

sizes of the allele fragments and validate corresponding on-chip results.

The quadruplex STR system included amelogenin and three Y-chromosome STR loci, DYS390, DYS393, and DYS439. Table 1 presents the PCR primers and associated dye labels, as well as the expected STR repeat numbers and amplicon lengths. The forward primers were labeled with energy-transfer dye cassettes developed in our group and described previously.[33] The 20-μL PCR mixture prepared for each experiment was composed of gold ST*R buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 160 μg/mL BSA, 200 μM each dNTP) (Promega), templates ranging from 0 to 50 copies in the 160-nL PCR chamber, primers, and FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). The corresponding primer concentrations in the singleplex PCR amplifications were 150 nM for amelogenin, 80 nM for DYS390, 120 nM for DYS393, and 180 nM for DYS439, respectively. In the multiplex PCR reactions, the primer concentrations were adjusted to 150 nM for amelogenin, 150 nM for DYS390, 120 nM for DYS393, and 180 nM for DYS439. The final DNA polymerase concentration was 0.2 unit/μL in all experiments except for the analyses of male DNA in female DNA background, where the concentration was increased to 0.4 unit/μL. The thermal cycling protocol was composed of initial activation of the Taq polymerase at 95 °C for 4 min, followed by a PCR cycle of 95 °C for 10 s, 58 °C for 60 s, 72 °C for 30 s, and a final extension step for 2 min at 72 °C. For the singleplex reactions, 32 cycles were employed while 35 cycles were used for the multiplex.

Following microchip PCR amplification, the CE separation channel was heated to 70 °C using the channel heater. After the microvalve adjacent to the sample reservoir was opened, the amplified sample was electrophoretically injected into the CE system toward the waste by applying an electric field of ~100 V/cm while floating the anode and cathode. A separation field of 250 V/cm was then applied between the cathode and anode. In the first 5 s of the separation, a backbiasing field of 80 V/cm was applied at the sample and waste, which were floated for the remainder of the separation. Raw electropherograms were processed with BaseFinder 4.0. Processing procedures include baseline adjustment, cross-talk analysis, and convolution filtering. After each run, the glass manifold was removed, the PDMS membrane was replaced, and channels and chambers were cleaned using piranha (7:3 H_2SO_4/H_2O_2) to prevent run-to-run carryover contamination.

RESULTS AND DISCUSSION

The quadruplex STR system for testing the portable four-color PCR−CE microsystem consists of the loci DYS390, DYS393, DYS439, and amelogenin. DYS390, DYS393, and DYS439 are members of the extended minimal haplotype loci, which play central roles in the current Y-STR DNA typing.[34] The haplotype diversity of these three loci is 0.9473 in the U.S. population. In addition to these three Y-STR loci, amelogenin, which codes for a protein found in tooth enamel, was employed. PCR amplification of this marker produces a 106-bp and a 112-bp amplicon from the X and Y chromosomes, respectively. Amelogenin is widely used for sex-typing and sample quality evaluation in the forensic community.[35] In our system, amelogenin serves as a positive control, providing important information about sample quality and amplification performance.

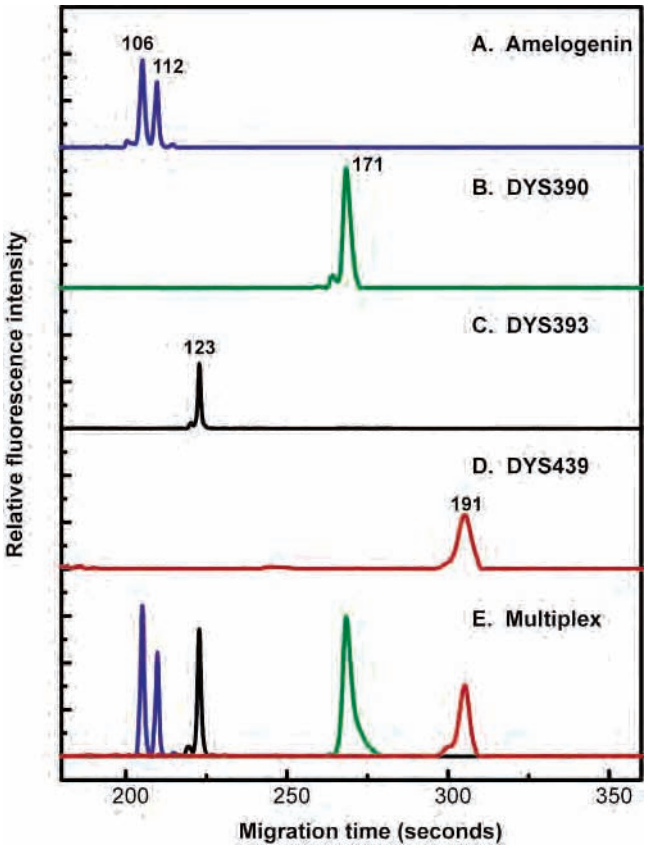

Figure 4. Singleplex and multiplex STR amplification performed on the PCR−CE microsystem. (A) Amplification of the amelogenin marker from male standard genomic DNA. A 106-bp X-chromosome and a 112-bp Y-chromosome amplicon labeled with FAM−FAM were amplified from 20 copies of the template with 32 PCR cycles. (B) DYS390 Y-chromosome amplicon (171 bp) labeled with FAM-R6G from standard male genomic DNA. (C) A 123-bp DYS393 amplicon from male standard genomic DNA labeled with FAM-TMR. (D) A 191-bp DYS439 amplicon labeled with FAM-ROX. (E) Multiplex PCR of all four loci from 50 template copies with 35 PCR cycles.

Singleplex and Multiplex STR Amplification. Singleplex amplifications on each locus were performed first to examine the functionality of the PCR−CE microsystem as well as the amplification performance of these DNA markers. In these PCR experiments, each DNA marker was amplified from 20 copies of 9948 male standard genomic DNA templates in the 160-nL PCR chamber with 32 PCR cycles. After thermal cycling, the PCR product was immediately injected and separated on the electrophoresis channel. An entire analysis was completed in 1.5 h. Panel A in Figure 4 presents an amplification of the amelogenin marker. A 106-bp X-chromosome and a 112-bp Y-chromosome amplicon labeled with FAM−FAM were observed, indicating that the template is male DNA as expected. Panel B presents an amplification and detection of the DYS390 locus, revealing a 171-bp

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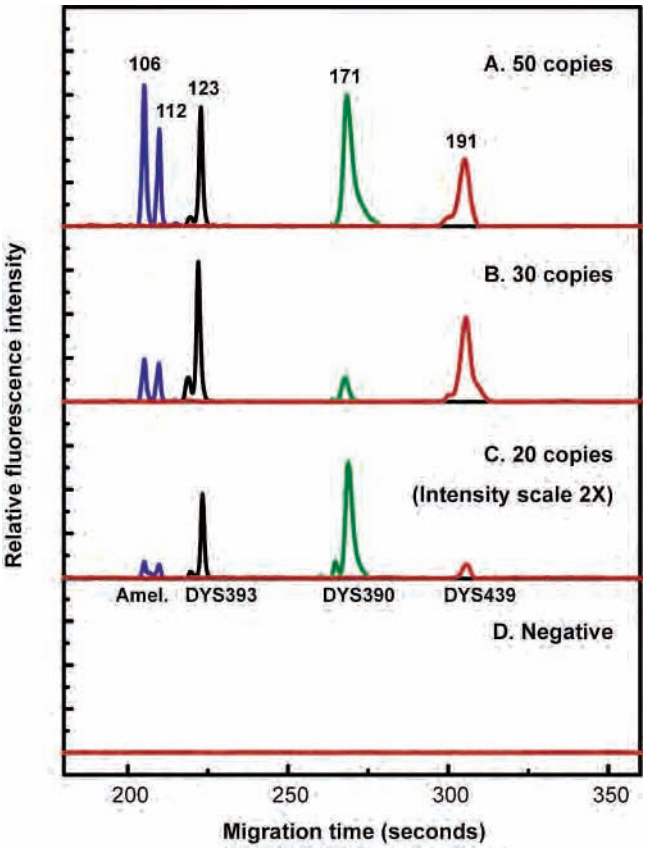

Figure 5. Limit of detection for multiplex analyses of 9948 male standard genomic DNA using the PCR−CE microdevice. PCR cycles were 35 in each case. The trace obtained from 20 template copies was enlarged twice for display. A negative control experiment was performed to confirm the absence of carryover.

amplicon labeled with FAM-R6G. Similarly, a 123-bp DYS393 amplicon labeled with FAM-TMR and a 191-bp DYS439 amplicon labeled with FAM-ROX were obtained, respectively, in panels C and D. With optimized primer concentrations (150 nM amelogenin, 80 nM DYS390, 120 nM DYS393, and 180 nM DYS439), each DNA marker demonstrated a similar amplification efficiency and good sensitivity.

Following successful amplifications on each locus individually, a multiplex PCR−CE experiment was carried out on this four-locus multiplex system. Starting template (50 copies of 9948 male standard genomic DNA) was loaded in the PCR chamber, and 35 PCR cycles were performed. Primer concentrations used in the multiplex system were adjusted slightly to maintain balanced peak intensities for each locus (150 nM for amelogenin, 150 nM for DYS390, 120 nM for DYS393, and 180 nM for DYS439). As shown in Figure 4 (panel E), all the peaks (106, 112, 123, 171, and 191 bp) were fully resolved and balanced. Compared with singleplex amplifications, multiplex STR amplifications exhibit lower amplicon yields due to competition between each locus. Therefore, both the initial template copy number (50 copies) and the PCR cycle number (35 cycles) were increased to compensate for this effect.

A limit-of-detection analysis for multiplex amplification of 9948 male standard genomic DNA was performed. Figure 5 presents results from a series of amplifications conducted from 0, 20, 30, and 50 copies of template in the PCR chamber with 35 PCR cycles. Even with only 20 copies of DNA template, the multiplex amplification still shows all the expected peaks in the electropherogram. An amplification from 10 copies was also performed; however, a complete profile was not obtained. The amplicon peak intensities are reduced and show more variability as the initial templates decrease from 50 to 20 copies. When the template copy numbers fall into the low copy number amplification range (<100 pg or <33 copies),[2] stochastic effects occur, and repeated amplifications of identical solutions exhibit fluctuations in peak intensity. Finally, it should be noted that the absence of any amplicons in the negative control (0 initial copies) demonstrates the effectiveness of the piranha cleaning conducted after each run.

Analysis of Forensic Casework. Samples obtained from forensic casework usually have lower amplification efficiency, due to PCR inhibitors, which remain with the DNA throughout the sample preparation process,[36,37] or due to DNA degradation by exposure to environmental elements or natural contaminants.[38] Here we selected two typical samples, one from an oral swab and the other from human bone, which were previously processed and analyzed by the Palm Beach County Sheriff's Office. Buccal cell collection with a cotton oral swab is often used in cases where reference samples from suspects or family members are needed to perform comparative DNA testing.[39] Human bone remains in forensic casework represent one of the most degraded biological materials for PCR-based DNA typing, since they are usually collected after a long period of exposure in a harsh environment, such as burial in soil.[40] Therefore, these two typical samples were chosen to test our integrated PCR−CE forensic system.

Four separate amplifications, including 9948 male and 9947A female standard genomic DNA, which serve as controls, and two casework samples from an oral swab and human bone, were conducted from 50 template copies with 35 PCR cycles. Panels A and B in Figure 6 present the PCR analyses conducted from male and female standard DNA, showing all the expected peaks with correct gender discrimination. Figure 6C presents an amplification and analysis of the DNA sample extracted from an oral swab. All the amplicons in four loci were successfully obtained, indicating the sample is male DNA. Figure 6D shows only one peak at 106 bp, corresponding to the successful amplification of female human bone DNA. Off-chip results using an ABI Prism 3100 confirmed the genders of these two samples and indicated that the amplicon lengths of the oral swab sample in DYS390 and DYS439 are 167 and 187 bp, one repeat less than those corresponding amplicons from 9948 standard DNA. These differences were also observed in the on-chip results, by aligning the profiles of the male standard DNA and the oral swab sample.

Mixture Analysis. The ability of our system to provide interpretable DNA amplification profiles, when a minute amount of male DNA is present in a high background of female DNA, is very critical, as this situation is often encountered in Y-STR forensic analysis.[6] Quadruplex amplification and detection was

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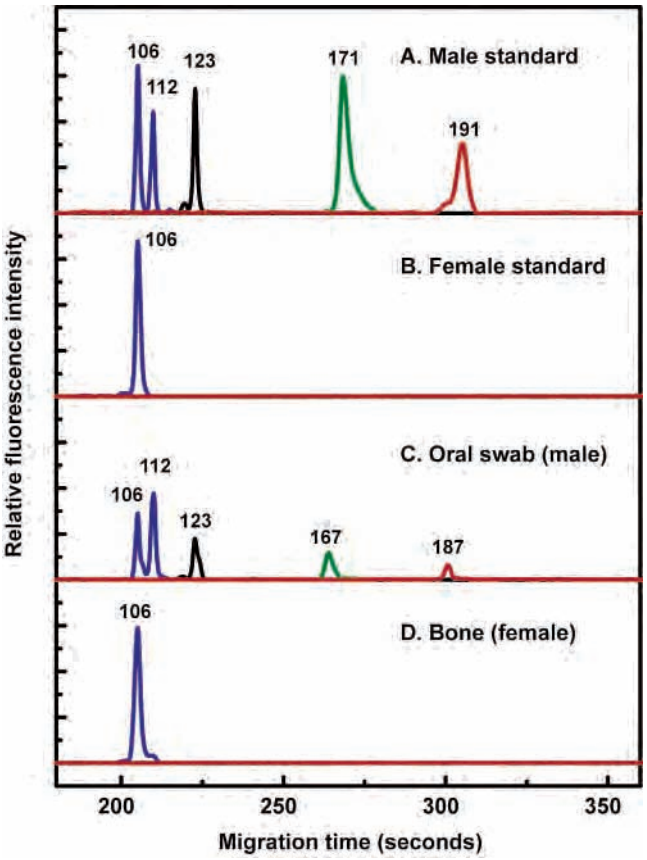

Figure 6. Multiplex STR forensic analysis using the PCR−CE microdevice conducted from standard genomic DNA and from samples extracted from an oral swab and human bone, respectively. In each case, 50 starting template copies and 35 PCR cycles were employed. (A) Analysis conducted from 9948 male standard DNA, showing the presence of all the amplicons on these four loci. (B) Analysis of 9947A female standard DNA, showing only the expected presence of the 106-bp X-chromosome peak. (C) Analysis conducted on genomic DNA extracted from an oral swab. All the expected peaks were observed, showing the source is male. (D) Analysis of a human bone sample. Only one 106-bp amplicon was detected, showing the source is female.

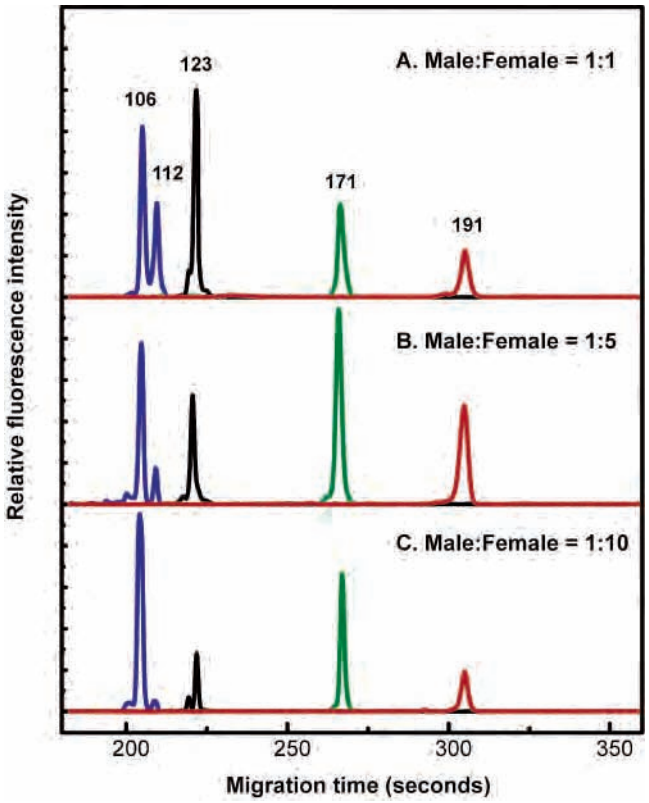

Figure 7. Multiplex STR analysis of male genomic DNA (50 copies) in the presence of a female genomic DNA background using the PCR−CE microsystem (35 PCR cycles). The template ratios of male-to-female range from 1:1 to 1:10. As the ratio increased, the 106-bp amelogenin amplicon from the X-chromosome became more and more dominant over the 112-bp Y-chromosome product.

carried out by mixing male and female standard genomic DNA together during the sample preparation. The male DNA in each run was maintained at 50 copies, while the female DNA was increased to achieve ratios of male-to-female genomic DNA of 1:1, 1:5, and 1:10, respectively, resulting in ratios of Y-to-X chromosomes of 1:3, 1:11, and 1:21. Since a high yield of the 106-bp X-chromosome product is expected to overwhelm the other Y-chromosome amplicons, the DNA polymerase concentration was increased from 0.2 to 0.4 unit/μL to ensure full amplification and to produce balanced profiles. The results of this experiment in Figure 7 show that, as the ratio increased, the 106-bp amplicon from X chromosome became more and more dominant over the 112-bp Y-chromosome product. The peak area ratios are roughly equal to the initial template ratios of Y-to-X chromosomes (1:3, 1:11, and 1:21). The other three Y-chromosome loci (DYS390, DYS393, and DYS439) were still fully amplified and balanced in each case. However, slight signal reductions were observed, due largely to the increase of the 106-bp X-chromosome amplicon, which used up most of the PCR resources. These data indicate that the system is capable of analyzing male DNA in the presence of a high female DNA background. Although the ratio could be lowered further in amplifications without the amelogenin marker, additional valuable information, such as the male-to-female DNA ratio, is obtained with this quadruplex system from the peak area ratio of the two peaks in amelogenin.

CONCLUSIONS

A fully integrated PCR−CE microdevice has been optimized for forensic analysis and combined with a new portable instrument including controls for chip operation and four-color fluorescence detection. This system was used to perform a quadruplex STR forensic analysis; the entire assay was finished in 1.5 h due to the rapid low-volume (160 nL) thermal cycling and integrated high-speed electrophoretic separation. The detection limit of this system for multiplex amplification of genomic DNA is as low as 20 copies in the PCR chamber. Two real-world forensic casework samples extracted from an oral swab and human bone were successfully analyzed, showing the practical application of this system. Finally, male genomic DNA was tested in the presence of excess female genomic DNA background. Intense balanced peaks were observed even at the male-to-female DNA ratio of 1:10.

This microdevice presents a first and significant step toward a fully integrated and portable system allowing highly sensitive, rapid STR analyses in a setting outside a forensic laboratory. For practical forensic applications in the future, a co-injection structure can be included in the microdevice to facilitate running sizing and allelic ladders,[26] and more STR loci should be included to improve

the discrimination power. Additionally, autosomal STR typing is under investigation to extend the application range of the portable microsystem. The integrated, high-speed and low-volume STR typing methods developed here will accelerate the forensic identification process and lower the assay cost, thereby reducing backlogs and advancing forensic DNA applications. Furthermore, our demonstration of successful STR analyses on a portable PCR−CE system validates the concept of point-of-analysis DNA typing in crime scene, mass disaster, or security checkpoint applications, where rapid on-site human identification is demanded.[5,41,42]

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