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

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USDJ Office of Justice Award Number: 2007-DN-BX-K142
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ABSTRACT

The overall goal of this project was to develop faster, more reliable, higher throughput, more sensitive and more 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 as well as through the NIJ grant awarded from 2004. These technologies include the use of more sensitive energy-transfer fluorescent dye labels, the development of microfabricated capillary array electrophoresis (μCAE) 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) optimized a portable microsystem that includes the steps of PCR amplification, sample and standard injection, separation and fluorescence detection in a single integrated microchip. Real-time STR analyses using a 9-plex STR kit, including sample collection, DNA extraction, STR analyses, and CODIS database search, were carried out with this device at a mock crime scene in collaboration with the Palm Beach County Sheriff's Office; (ii) developed an integrated STR sample cleanup, concentration method that employs a photopolymerized streptavidin-gel capture chemistry coupled to a simple, improved direct injector geometry to achieve higher fluorescence signals (~19-fold) and sensitivity (25 copies of DNA) for STR typing; (iii) integrated this inline capture injector into our previous μCAE microsystem to develop a 12-lane capture-CAE microsystem with automated operation for high-throughput and high-sensitivity STR sample analysis; (iv) demonstrated the integration of sequence-specific DNA extraction using a magnetic bead capture structure and the improved post-PCR capture inline injector into the PCR-CE system to form a fully integrated microdevice for rapid forensic STR analysis; (v) constructed the 2nd generation bench-top rotary μCAE scanner, the Multi-channel Capillary Array Electrophoresis Portable Analyzer (McCAEPS), with reduced size and operational complexity for integrated STR analysis.

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Contents

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EXECUTIVE SUMMARY

1. Portable Genetic Analyzer for Rapid On-Site STR Analysis

We first explored the concept of point-of-analysis forensic human identification by developing an improved integrated PCR-CE microdevice and typing method capable of conducting real-time forensic STR analysis, and by using this system at a mock crime scene. Real-time DNA analyses were carried out in collaboration with the Palm Beach County Sheriff's Office (PBSO). This work was disseminated in *Forensic Science International: Genetics*.

The PCR-CE microchip includes a 160-nL PCR reactor with a heater and a four-point resistance temperature detector (RTD) for thermal cycling, two poly-(dimethysiloxane) (PDMS) microvalves for microfluidic control, a coinjector, and a 7-cm-long CE separation channel. For proof of concept, a 9-plex autosomal STR typing system, consisting of Amelogenin, and eight STR loci was employed to test our system. The microchip is prepared for PCR-CE analysis following the same protocol as described previously.[1] The modified thermal cycling time is about 2 hr, and the CE separation takes about 8 min.

Allelic ladder separation. The CE separation of a preconcentrated PowerPlex® 16 allelic ladder (4×) and an ET-550 sizing ladder using this microdevice was completed in 8 min, which is approximately 20 min faster than the single-capillary ABI 310 instrument. The TH01 9.3 and 10 alleles were distinguished with a resolution of 0.45.

Standard DNA test. The 9-plex autosomal STR amplification and separation on the portable PCR-CE microsystem was demonstrated using standard female 9947A and male 9948 genomic DNA. The run-to-run standard deviations (n=3) of the allele sizes were calculated from the traces of 9947A and 9948 DNA obtained in the standard DNA test. All the alleles can be sized correctly with a standard deviation $\leq$ 0.8 bp.

Limit of detection study. The sensitivity of the portable PCR-CE microsystem was evaluated using the 9-plex STR samples amplified from serially diluted 9947A standard DNA. Complete profiles can be reproducibly obtained from 100 template copies.

DNA analysis at a mock crime scene. Real-time STR analyses were carried out at a mock crime scene prepared by the PBSO. The PBSO Forensic Biology Unit prepared blood stain samples on paper towels and cloth, representing a male and a female victim,

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and a male suspect, from known individuals with documented DNA profiles. A PBSO Mobile Command Unit (MCU) was deployed to the mock crime scene, as shown in Figure 1. The crime scene was set up in a pavilion at Lake Lytal Park, Palm Beach, FL by PBSO crime scene investigators.

Figure 1. (A and B) Photographs of the mock crime scene investigated by PBSO in West Palm Beach, FL. Three blood stains on cloth or paper towels were laid out in the mock crime scene. (C) The entire DNA analysis was conducted in a mobile command unit provided by PBSO. (D) The portable forensic analysis instrument set up in the mobile command unit.

All DNA samples were extracted using the Maxwell 16® instrument with DNA IQ™ Casework Sample Kit (Promega) following the manufacturer's protocol. After the Maxwell run was completed in 30 min, DNA extracts in the final tubes were concentrated using Microcon columns to a final volume of 7 μL. The PCR cocktail (4 μL of DNA sample in 10 μL of PCR cocktail) was loaded into the PCR reactor with a target

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concentration of 300 template copies in the reactor. Figure 2 presents the electropherograms of the three samples analyzed with the 9-plex STR system.

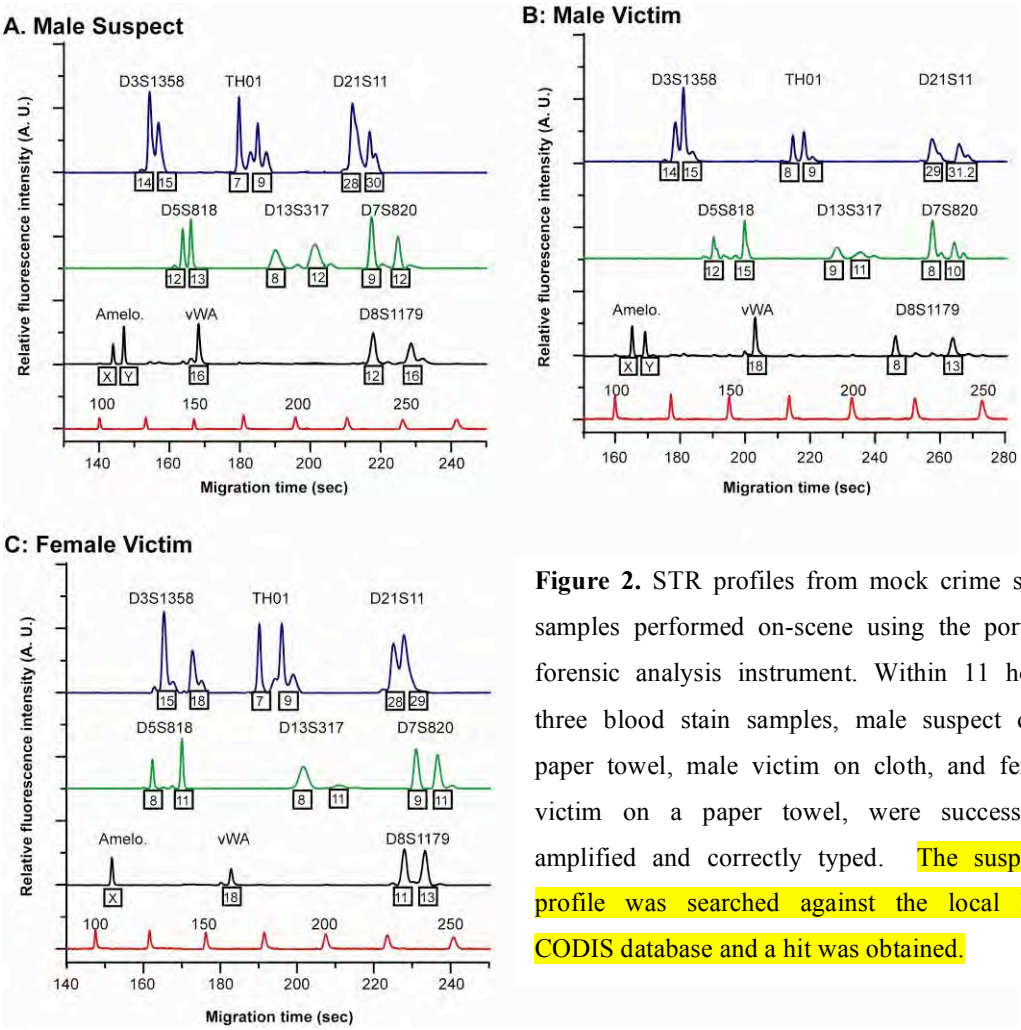

Figure 2. STR profiles from mock crime scene samples performed on-scene using the portable forensic analysis instrument. Within 11 hours, three blood stain samples, male suspect on a paper towel, male victim on cloth, and female victim on a paper towel, were successfully amplified and correctly typed. The suspect's profile was searched against the local level CODIS database and a hit was obtained.

This work establishes the feasibility of using our portable PCR-CE microdevice for real-time forensic STR analysis at a crime scene or a security location. It presents some interesting topics for forensic policy and practice. The on-site human identification could be used for real-time analysis at crime scenes to facilitate case detectives' investigation before the suspect has fled the area or destroyed secondary evidence. Second, in states that require DNA samples at arrest, the ability to rapidly type an arrestee before release on bail might generate probable cause to hold the individual because of hits on other earlier criminal activities. In the future, a sample purification

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structure integrated with on-chip PCR and CE would be desirable to facilitate directly STR analysis from real-world samples containing PCR inhibitors. Furthermore, an affinity-capture-based in-line injector in place of the inefficient cross-injector is also needed to achieve more sensitive analysis.

*2. Development of **Single-Channel** Sample Cleanup and CE Device*

Current STR analysis by CE bypasses the post-PCR sample cleanup step in order to save time and cost. While current methods have a sample dilution step which dilutes the high salt PCR samples, resulting in an acceptable separation analysis, the injection has poor efficiency due to sample dilution and is still biased against larger loci. Additionally, the cross-injector geometry employed in the conventional CE microchips requires delicate timing and electric field balance. To address these issues, we developed an integrated STR sample cleanup, concentration method that employs a photopolymerized streptavidin-gel capture chemistry (Figure 3A) coupled to a simple, improved direct injector geometry (Figure 3B&C) to achieve higher fluorescence signals and sensitivity for DNA typing. Results of this work and the technical details were disseminated in *Analytical Chemistry*.

The following summarizes results from our evaluation of this new integrated sample processing method and chip.

Integrated capture-μCE enhances fluorescence intensity. For a diluted sample, the fluorescence intensity of the TH01 alleles is enhanced by at least 10 fold compared to cross injection. When an undiluted PCR sample is used, the signal intensity is enhanced by ~ 50 fold. Also, DNA fragments that differ by a single base can be differentiated with an average resolution (Rs) of 0.65 (σ = 0.1) and a valley value of (Vv) of 50% (σ = 3%).

Integrated streptavidin–μCE analysis of STR samples. A biotin-modified 9-plex STR typing kit was constructed and balanced using primer sequences and fluorescent dye-labels found in the PowerPlex® 16 kit. A 14-fold increase in fluorescence intensity was observed for the diluted 9-plex reactions amplified from 0.5 ng of standard human genomic DNA using the streptavidin-μCE analyses compared to separations with the cross-injector device. When pure amplified PCR samples were used, the fluorescence intensity was increased by ~19X.

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Limit of Detection. A sensitivity study was carried out to evaluate the detection limit of the system using biotin-modified 9-plex PCR reactions amplified from serially diluted 9948 standard genomic DNA. All 15 expected STR alleles were successfully and reproducibly detected (defined as S/N ≥ 3) with as few as 25 copies of DNA input.

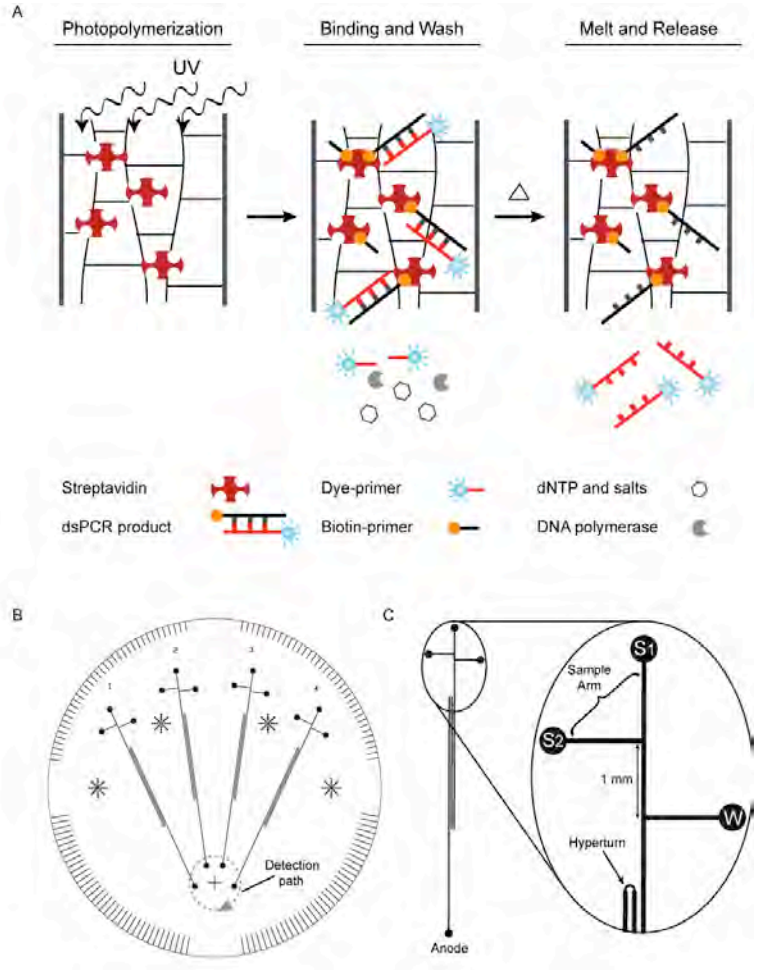

Figure 3. (A) Schematic of the streptavidin-gel capture method for purifying and concentrating biotin-modified PCR products. One primer labeled with fluorescent dye and one with biotin are used to generate PCR products. The PCR reaction is electrophoresed through the cross-linked polyacrylamide get network where the ds-DNA products bind strongly to the streptavidin gel. Unreacted PCR materials are washed off and then the fluorescently labeled DNA strand is thermally released from its complementary captured strand for electrophoresis down the separation column. (B) Design of the 4-channel integrated capture-μCE array.

Degraded DNA typing. Four simulated degraded DNA samples were prepared and provided by the VDFS to test the ability of the streptavidin capture–μCE method to

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improve analyses of compromised DNA samples. Blood samples collected from two individuals were each exposed to 56 and 80 °C for 3 months and stored for six years to simulate degradation. Since our microchip-based CE system provides similar performance to that of conventional CE systems in terms of sensitivity, we compared the results obtained by the capture–μCE device with those using the cross injection. For DNA samples that resulted in no alleles (Fig. 4A) or a few alleles (Fig. 4B) with cross injection, ~ 33% and ~71% more allelic markers were obtained, respectively, using the streptavidin capture–μCE method.

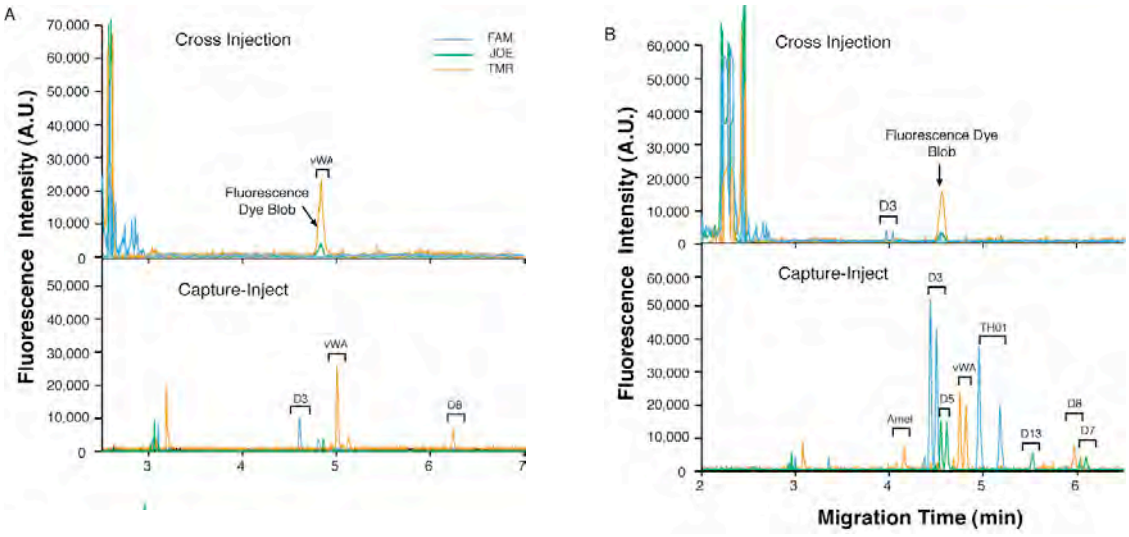

Figure 4. Analyses of PCR reactions amplified from two degraded DNA samples. (A) For sample A2, no alleles were detected using the cross injection method and a dye blob is observed overlapping with a putative vWA peak. Analysis with the capture-μCE device and method produced three alleles at D3, vWA and D8 loci with fluorescent counts of 10K, 30K and 5K counts. (B) For sample B2, two D3 alleles were detected using the cross injection method and a dye blob observed overlapping with the putative vWA alleles. Analysis with the capture–μCE process yielded ~ 10X higher fluorescent intensity for the two D3 alleles and 10 additional alleles with signals from 5K to 30K fluorescent counts while eliminating dye blobs and primer signals.

The development of the photopolymerizable streptavidin-gel capture method coupled with a simple injector geometry for rapid purification and electrophoretic separation of STR enables the capture of additional allele data from degraded DNA samples, and helps improve forensic lab efficiency by dramatically increasing the fluorescence signals for low-level DNA samples. This inline capture injection can be integrated with our multi-lane μCAE or on-chip PCR method, leading to the realization

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of high-throughput, and high-performance genetic analysis systems for a variety of applications.

*3. Development of **multi-lane** Capture-CAE microsystem*

The capture inline injector can be incorporated into our previous microfabricated capillary array electrophoresis (μCAE) microsystem[2] to achieve the capability of high-throughput STR sample analysis. As shown in Figure 5, we developed a 12-lane capture-CAE chip using the same chip layout as those of our previous μCAE devices. This work has been submitted to *Forensic Science International: Genetics.*

The following summarizes results from our evaluation of the capture-CAE microdevice, including the capability of high-throughput STR typing, comparison with conventional cross injection, limit of detection, and touch evidence analysis.

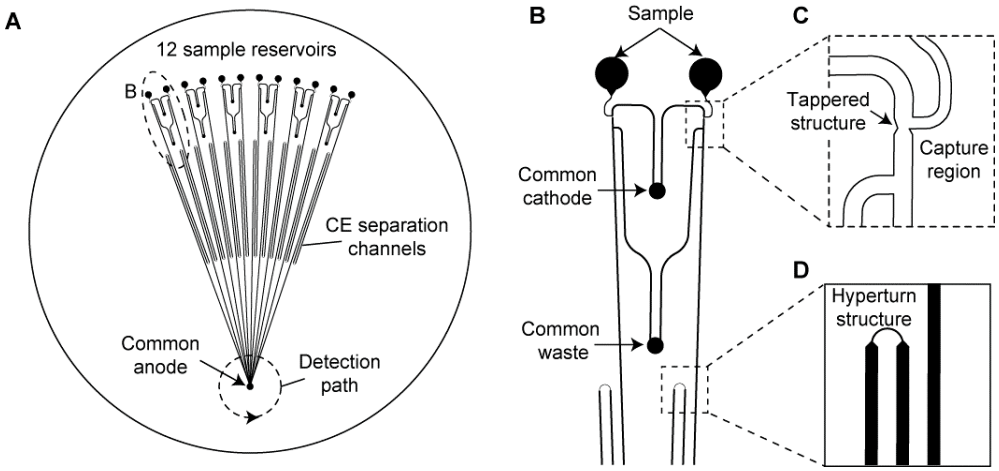

Figure 5. The schematic of the 12-lane capture-CAE microdevice. (A) A total of 12 electrophoretic separation channels coupled with capture gel inline injectors arranged on a 4" glass wafer, forming six doublets. (B) Each doublet includes two capture gel inline injectors and two sample wells sharing one cathode and one waste well. (C) The expanded view of the capture region. (D) The expanded view of the hyper-turn structure.

High-throughput STR analysis. We analyzed 9-plex STR samples amplified from 50 copies of 9947A standard genomic DNA on the 12-lane microdevice to test the chip design and the operation protocol for high-throughput forensic STR typing. As shown in Figure 6, STR profiles were successfully obtained from all the 12 lanes in 30 min. The average percentage standard deviation of the allele signal-to-noise (S/N) ratios is 18.8 % and the average standard deviation of the allele migration time is 9.8 sec.

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Comparison with conventional cross injection. In this study, the same STR samples amplified from 50 copies of 9948 standard genomic DNA were analyzed by using both cross injection and capture inline injection methods on the capture-CAE microchip under the same detection settings. The allele S/N ratios in the 9-plex STR profile obtained using the capture inline injection was improved 12.1±1.8 fold over the cross injection. The significant reduction of primer peaks is especially beneficial for incorporating smaller loci into the multiplex STR system by opening up space in the electropherogram.

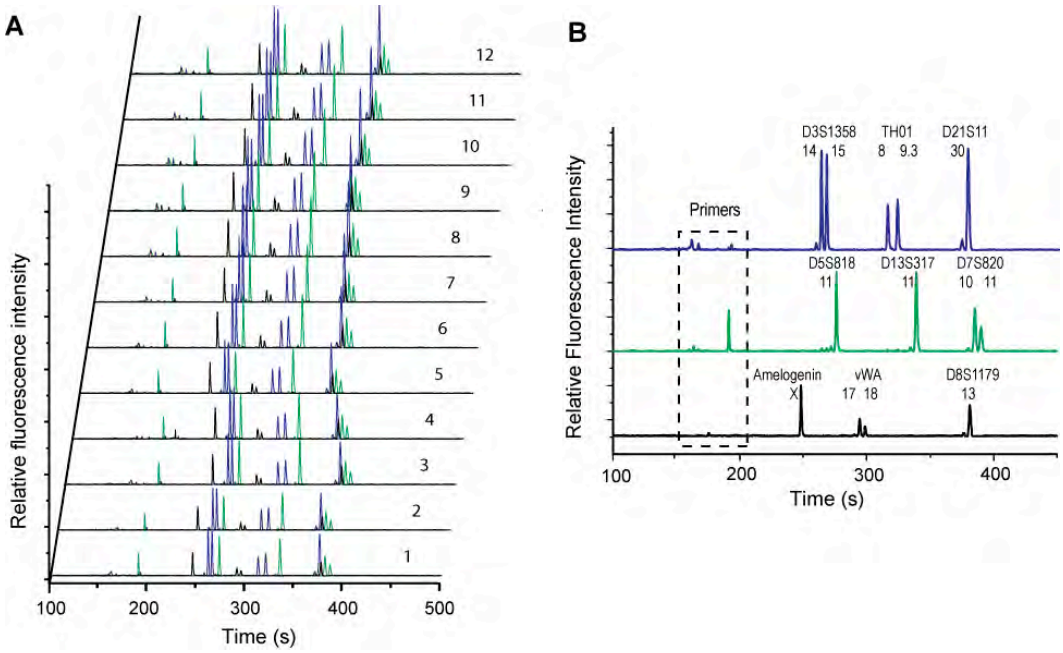

Figure 6. (A) Electropherograms from 12 lanes in a single run. All the traces are plotted in the same signal intensity scale demonstrating that all 12 channels can provide similar performance. (B) Example of one of the typical 9-plex STR traces obtained from the 12-lane μCapture-CAE microsystem. The dramatically reduced primer peaks show the effectiveness of our sample cleanup procedure.

Limit of detection test. A sensitivity study was carried out to evaluate the detection limit of the capture-μCAE microsystem by using 9-plex STR samples amplified from serially diluted 9947A standard genomic DNA. All 13 expected STR alleles were successfully and reproducibly detected with as few as 35 copies of DNA input.

Touch evidence analysis. Touch evidence samples recovered from unfired bullet cartridges were prepared by the VDSF and shipped to Berkeley for analyzing on the 12-lane capture-CAE microsystem. In total, three samples, the concentrations of which are

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characterized as 4.1, 4.6, and 4.8 pg/μL, were tested on the capture-μCAE system. 53%, 71%, and 59% of the 9-plex STR profiles were successfully obtained by using the sample cleanup, concentration and analysis method on the capture-CAE device. As a comparison, when the same samples were analyzed using the cross injection method under the same experiment settings, only blank profiles were observed.

The capture-CAE microsystem provides a reliable and robust platform for forensic STR typing of LCN and degraded DNA due to its seamless integration of analytical steps, automated operation process, and 100% efficient sample analysis. This system has the potential to become a routine way to reliably analyze DNA samples in forensic investigations. In the future, the development of UV exposure systems for rapid photopolymerization at multiple spots would enable a 96-lane capture-CAE device without major modification. Additionally, this inline capture injection process can be incorporated into the on-chip PCR-CE system, leading to the realization of a highly sensitive, fully integrated system to explore more challenging studies in forensics, such as STR typing from single cells.

4. Development of integrated template capture chemistries and methods

The integrated PCR-CE microdevice developed previously falls short in its ability to analyze real-world samples containing PCR inhibitors.[3] To address this problem, a sequence-specific DNA extraction and an improved post-PCR capture inline injection structure were successfully integrated into the PCR-CE system to form a fully integrated microdevice for forensic STR analysis. A fluidized bed of streptavidin-coated magnetic beads immobilized in the channels[4, 5] captures the conjugates of biotin-labeled oligonucleotide probes and genomic DNA fragments containing the STR locus sequences. After DNA capture, the bead-DNA conjugates are pumped to a PCR reactor for 9-plex STR amplification. The resulting biotin-labeled PCR products are electrophoretically driven through a streptavidin-modified capture gel where they are bound and concentrated into a narrow injection plug, followed by thermal release for CE separation. We are preparing a manuscript on this work for publication in *Analytical Chemistry*.

Microdevice design. The microdevice shown in Figure 7 contains two identical genetic analysis systems forming a symmetrical doublet on a 4-inch glass wafer. Each

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analytical system includes a PDMS micropump and two PDMS microvalves for fluidic control,[6] a 4-cm-long bead capture structure with a system of bifurcating channels for DNA template capture,[4] a 250-nL PCR chamber with a microfabricated heater and a RTD for PCR thermal cycling, a 500-µm-long double-T channel junction with a tapered structure for post-PCR cleanup and inline injection, and a 14-cm-long channel for CE separation.

STR typing and DNA capture probes. A 9-plex autosomal STR typing system developed previously was employed to test the system. To enable the post-PCR cleanup and inline injection, the unlabeled primers were replaced with biotin-labeled primers (IDT, Coralville, IA). The biotin-labeled primers were also employed as capture probes for DNA template capture prior to PCR.

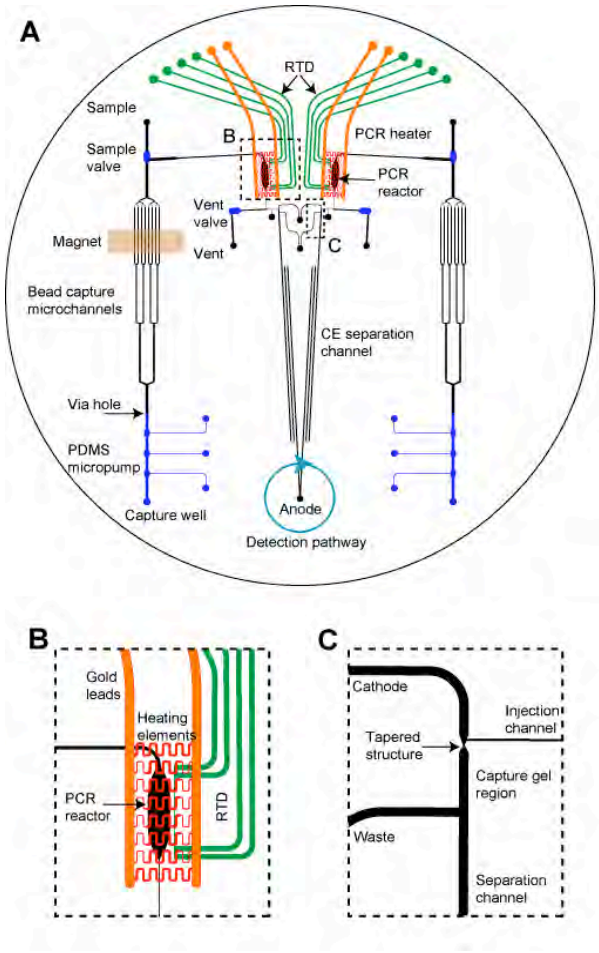

Figure 7. The design of the fully integrated microdevice for forensic STR analysis. (A) Mask design for the microchip capable of performing DNA template capture, PCR, capture inline injection and CE separation. (B) Expanded view of the PCR chamber and the microfabricated heater and RTD. (C) Expanded view of the capture inline injector with a tapered structure.

DNA sample preparation. Standard genomic DNA 9947A, 9948 and K562 were purchased from Promega and diluted in deionized water (DI water). To prepare DNA samples for on-chip template capture, 20 µL DNA samples were first heated at 95 ^{o}C for 15 min in a PTC-200 thermocycler. After mixing the fragmented DNA with 25 uL of 20×

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sodium saline citrate (SSC) buffer and 5 uL of 10× capture probe mixture, the solution was further heated to 95 ^{o}C for 5 min, followed by incubation at 50 ^{o}C for 20 min to allow DNA hybridization between the biotin-labeled capture probes and the target DNA.

Microchip operation. Following the photopolymerization of the capture gel plugs in the chip using the method developed previously,[7] a separation matrix (5% LPA with 8 M Urea in 1× TTE) is loaded from the anode to the waste and from the cathode to the coinjection reservoir to form a matrix-capture-matrix gel sandwich structure in the capture inline injection regions. SSC (10×) is loaded into the bead capture channels while keeping the vent microvalve closed.

Total 5 μL Dynabeads (Invitrogen, Carlsbad, CA) (~1.3×10^{6} beads) are driven into the capture structure using the on-chip micropump and immobilized in the microchannels using a nickelplated neodymium magnet (All Electronics, Van Nuys, CA). DNA solution (20 μL) containing fragmented genomic DNA and capture probes is then pumped through the capture structure using a 9-step pumping protocol with three "flutter" steps.[4, 5] After washing with 10 μL PCR cocktail, the beads are then pumped into the PCR chamber by placing the magnet above the reactor. PCR thermal cycling starts with an initial activation of the Taq polymerase at 95 ^{o}C for 4 min. For the 32 PCR cycles, the temperature is held at 94 ^{o}C for 10 s denaturing, then ramped to 58 ^{o}C for 20 s annealing, and then to 70 ^{o}C for 30 s extension. Finally, a post extension step is performed at 70 ^{o}C for 5 min. Total PCR time is 40 min.

To perform the purification, the biotin-labeled PCR products are electrophoretically injected using an electrical field of 25 V/cm from the PCR chamber to the waste well through the capture gel plug, where the products are bound via the biotin-streptavidin interaction to form a tightly concentrated plug. After washing, the fluorescently labeled DNA strands retained in the capture gel are then thermally released into the separation channel by heating the whole chip to 67 ^{o}C and applying an electrical field of 250 V/cm towards the anode.

Genomic DNA digestion. To enable the sequence-specific DNA capture, genomic DNA with high molecular weight must be fragmented into an appropriate size range which can be captured by magnetic beads while still providing intact templates for

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subsequent PCR amplification. Heating DNA samples at 95 °C for 20 min is sufficient to fragment genomic DNA into a size range of 1-4 kb.

Sequence-specific DNA capture. The evaluation of the efficiency of the DNA template capture was performed on the glass microchip which only contains bead capture microchannels coupled with a PDMS micropump. The capture efficiencies for these 9 STR loci are in a range of 3.7 - 7.0 % with an average of 5.4 ± 1.3 %. Although this efficiency is lower than those of many stand-alone DNA extraction microdevices developed previously,[8, 9] the efficient transfer of the captured DNA to the PCR reactor compensates for this drawback.

Another advantage provided by sequence-specific DNA extraction is the improved PCR efficiency due to the complete removal of background DNA sequences. We conducted a comparison between 9-plex STR amplifications from 1 ng of whole genomic DNA (9948) and from ~ 1 ng DNA fragments purified from genomic DNA by sequence-specific DNA capture. The PCR amplifications were performed in a conventional thermal cycler with a PCR protocol which includes 10-s denaturation at 94 °C, 20-s annealing at 58 °C, and 30-s extension at 70 °C. Full STR profiles can be obtained from the purified DNA, but the profiles from the whole genomic DNA experienced dropout of the TH01 9.3 allele. This comparison demonstrates the effectiveness of sequence-specific DNA purification for improving STR amplification.

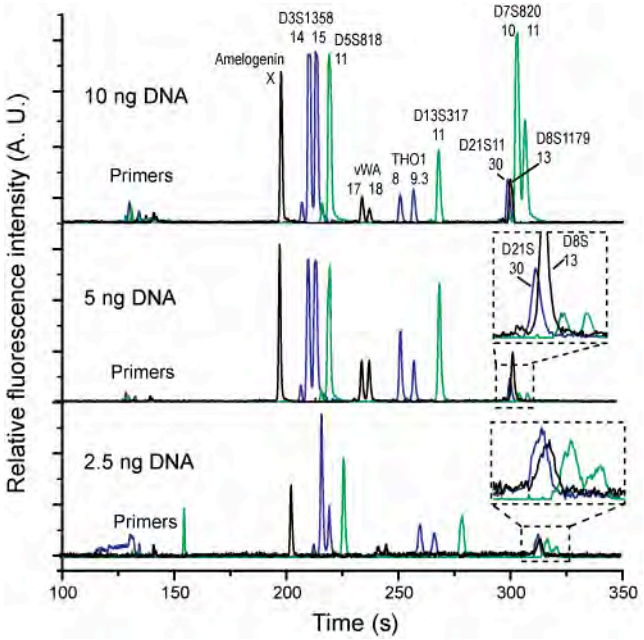

Figure 8. The limit of detection test of the microsystem using serially diluted female 9947A DNA. With only 2.5 ng DNA input, full DNA profiles can be obtained successfully.

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Limit of detection. DNA template capture, 9-plex autosomal STR amplification, post-PCR cleanup, capture inline injection, and CE separation on the fully integrated microsystem were tested using serially diluted female 9947A genomic DNA. As shown in Figure 8, full DNA profiles can be obtained with as few as 2.5 ng input DNA. Considering the average 5.4% capture efficiency of the sequence-specific purification, about 45 copies of DNA were purified from the 2.5 ng input and loaded into the PCR chamber for the following amplification, post-PCR cleanup, inline injection and CE detection. The detection limit of 45 copies for PCR amplification is already approaching to the low-copy-number range (≤ 100 pg or 33 copies of each locus). The total analysis time of this assay on the microsystem is about 2 hours and 35 min, at least 3-4 hours shorter than that using conventional method.

In conclusion, a fully integrated microdevice capable of performing DNA purification, PCR, post-PCR cleanup, and CE separation using a compact detection and control instrument and chip was successfully developed for rapid forensic STR analysis. This study is a significant step towards a fully integrated and portable forensic analysis system for rapid real-time human identification at crime scenes or other point-of-analysis situations.

5. Development of 2nd Generation μCAE Scanner

All of our work summarized above was supported by the development of a 2nd generation bench-top rotary μCAE scanner with reduced size and operational complexity for integrated STR analysis. The Multi-channel Capillary Array Electrophoresis Portable Analyzer (McCAEPS) is shown in Figure 9. The instrument was developed to perform μCAE analysis alone,[2, 10] cleanup and separation of off-chip amplified samples,[7] on-chip PCR and electrophoretic analysis,[1, 3] as well as fully integrated sample-in-answer-out assays in the future. Results of this work and the technical details are being prepared for submission to *Review of Scientific Instruments.*

The instrument contains a confocal optical system with a rotating objective for detecting four different fluorescence signals, pneumatics for control of on-chip microvalves and micropumps,[6] four PCR temperature control systems,[1] four high voltage power supplies and one grounded electrode for electrophoresis. The instrument has

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dimensions 12 × 12 × 8 in. with a total weight of 28 lbs, and it can be used as either a bench-top or portable instrument. To verify the performance of this instrument, we conducted the following assays:

Limit of detection and capillary electrophoresis. Fluorescein dye with a concentration range of 0.05-1 nM (pH 8) was separated on a 96-lane μCAE microdevice.[2] The detection limit of this system is determined as ~20 pM fluorescein with a S/N of 2. The separations of STR samples amplified off-chip using the 96-lane μCAE microdevice also produced fully resolved DNA profiles.

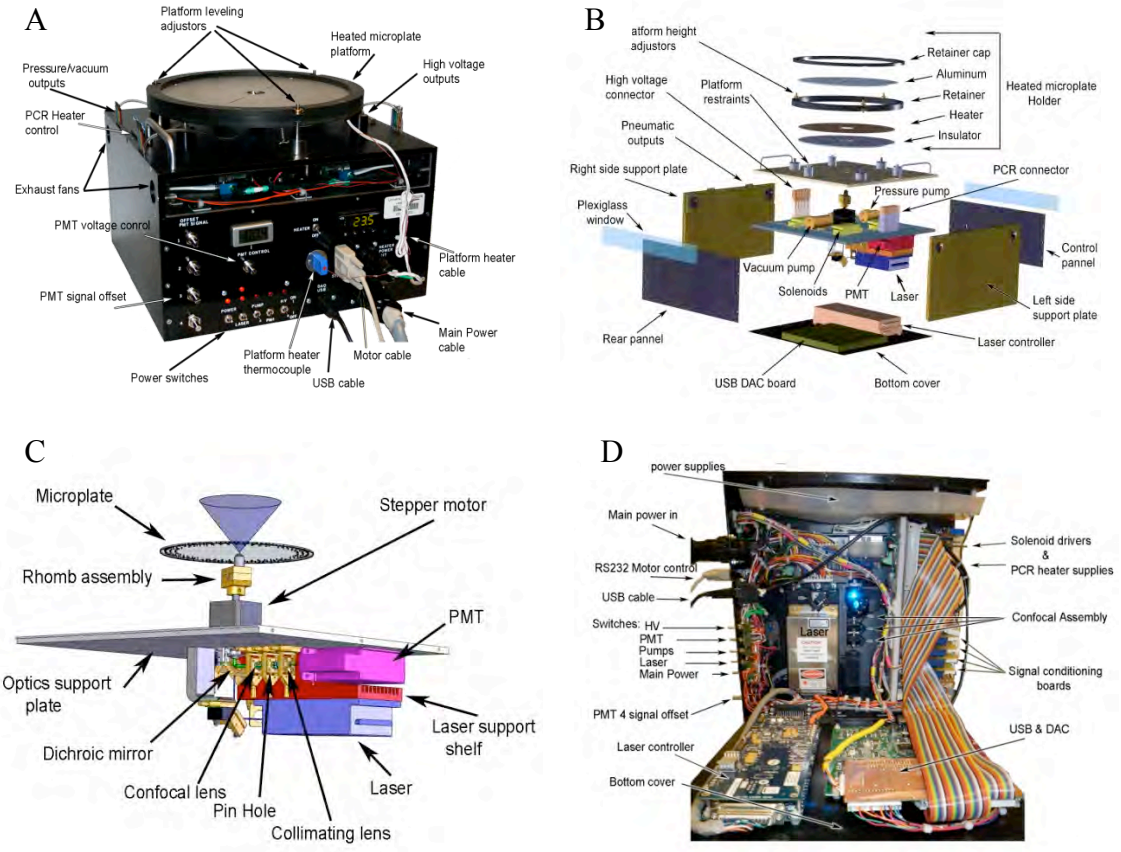

Figure 9. (A) Photograph of the McCAEPS. (B) Exploded view of the instrument. (C) Schematic of the 4-color confocal detection system with a rotary objective. (D) Bottom view of the open instrument.

Integrated PCR and electrophoresis. We performed an integrated PCR and capillary electrophoresis test using an integrated PCR-CE microdevice.[3] In this study, 100 copies of 9948 standard DNA mixed with PCR reagents were loaded into the PCR chamber for thermal cycling. The temperature ramp rates can reach 16 °C/s for heating

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and 13.8 °C/s for cooling. The 9948 DNA profiles obtained by this microsystem prove that the McCAEPS can be used as a general platform for integrated microfluidic devices.

In conclusion, we have developed a portable, self-contained instrument for control and detection of high-throughput integrated microfluidic microdevices. Several unique features, including high-throughput detection system, four temperature control units, and 28-solenoid-valve system, make this instrument a versatile platform for genetic analysis.

Dissemination

We have written or published five papers and made 5 conference presentations on this work. See complete listing of these activities after the Technical Report.

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DETAILED TECHNICAL REPORT

I. Introduction

Driven by the escalating backlog of crime scene evidence and the rising number of items submitted for DNA testing, forensic scientists are looking for techniques to automate the steps of forensic DNA analysis to make it faster, better, and cheaper.[1] However, a unique challenge faced by forensic laboratories is that DNA evidence are obtained from widely varying DNA 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 enough to accommodate the great disparity in DNA quality and quantity. While the utilization of automated capillary electrophoresis for short tandem repeat (STR) analysis has been in place in forensic laboratories for several years[2-6], the application of robotic DNA extraction[7] and quantitation[8-10] has just recently been implemented. It is also desirable to develop a thermocycler and DNA analysis system that can substantially reduce the amplification time 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 what is extensively used in forensic labs is the ABI 3100 Capillary DNA Sequencer that contains only 16 capillaries. [11, 12] In order to complete analysis of a single tray of 96 samples, it still takes many hours for electrophoresis and data capture. Even the most recent ABI 3500xl CE system with 24 capillaries still needs hours to finish 96 samples. A generic problem with capillary array separation systems is the inefficient injection (<1%) of STR fragments when injecting from high salt PCR solutions using the standard sample preparation protocol. This inefficiency is a disadvantage when performing LCN or degraded DNA typing. Neither the ABI capillary electrophoresis systems nor the Miraibio FMBIO® gel imaging systems sufficiently combine throughput with automation so that data are captured and analyzed in the most efficient, time saving manner.

Another key issue in forensic laboratories is the analysis of "touch" or low copy number (LCN) evidence and degraded DNA which usually contain very little DNA.[13, 14] A number of well documented problems in low copy number (LCN) profiling[15, 16] can

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arise including allele "drop-in", allelic drop-out and heterozygous peak imbalance.[17, 18] Reduced volume amplification reactions can increase sensitivity, but they too are subject to the increased appearance of drop-in alleles.[18-20] Equally important is the problem of typing degraded DNA from which only partial profiles are recovered due to highly fragmented DNA. Some of the major efforts to address these challenges have focused on reducing the size of STR alleles (> 200 bp).[21-25]

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 the integrated chip, 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 contamination.

Many of these needs were critical issues in the Human Genome Project a decade ago and were conquered through the development of improved, high-throughput capillary array electrophoretic (CAE) separation systems, through the development of better energy transfer dye labeling systems with 2- to 8-fold sensitivity enhancements and through the utilization of conventional but very useful macro-robotic systems for sample transfer. 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.[26] 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 (PBSO) led the eventual technology transfer and validation of a prototype μCAE instrument at VDFS.[27] 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.

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In order to fully take the benefits provided by microfabrication technology, sample preparation steps, such as PCR, should be integrated into the CE chips. The first demonstration of integrated PCR-based sample preparation and CE analysis (PCR-CE) on a chip was performed at Berkeley by our group in 1996.[28] Since then, others have demonstrated microfabricated PCR devices in silicon,[29, 30] glass,[31, 32] and glass capillaries.[33] Thermal cycling strategies include cycling a stationary fluid in a reactor [31-36] and continuous-flow systems in which the fluid is shuttled between three static temperature zones.[37, 38] Heating methods vary from direct contact methods in which the heating element is attached to the device to non-contact heating mediated by, for example, infrared exposure.[35, 39, 40] Some drawbacks to these methods include high starting template concentrations[37], inability to integrate into a fluorescence detection system,[37] or a relatively large sample volume.[31, 32] Few of these approaches are sufficiently reliable and scaleable to be incorporated into complex multichannel systems for STR analysis.

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,[41] (ii) the development of precisely microfabricated and reliable temperature sensors and integrated heater systems,[42] and (iii) the development of integrated oligonucleotide-gel capture matrices for PCR product purification.[43] With previous NIJ support (2004-2007), we developed an integrated PCR-CE microdevice together with a portable instrument which contains a 4-color confocal detection system and all the necessary electronic and pneumatic components for forensic DNA amplification and CE separation.[44, 45] Mini-Y STR typing was successfully performed on this microsystem, demonstrating the benefits provided by miniaturized and integrated process, such as high speed, less sample consumption, and portability. Only the IR thermal cycler system by Landers has been similarly extended to perform complex sample-to-read analysis of infectious disease.[40] 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 for addressing the needs of forensic analysis.

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II. Research Methods, Findings and Conclusions

1. Portable Genetic Analyzer for Rapid On-Site STR Analysis

We have previously developed and evaluated a portable STR analyzer for forensic DNA typing by performing 4-plex mini-Y chromosome STR analysis,[3] establishing the feasibility of conducting STR analysis on an integrated microdevice. We now further explore the concept of point-of-analysis forensic human identification by developing an improved integrated PCR-CE microdevice and typing method capable of conducting real-time forensic STR analysis, and by using this system at a mock crime scene. To enhance the discrimination power, a 9-plex autosomal STR typing system is constructed with amelogenin, a sex-typing marker, and eight CODIS core STR loci. Prior to the field trial, the entire analytical process was evaluated to optimize the amplification efficiency and separation resolution as well as the sizing calibration accuracy using commercial genomic DNA controls. Real-time DNA analyses at a mock crime scene are carried out in collaboration with the Palm Beach County Sheriff's Office (PBSO). This work was disseminated in *Forensic Science International: Genetics*.[45]

Microdevice design. The design of the four-layer PCR-CE microdevice has been modified from that previously developed in our group. The PCR-CE microchip (Figure 1) includes a 160-nL PCR reactor with a heater and a four-point resistance temperature detector (RTD) for thermal cycling, two poly(dimethysiloxane) (PDMS) microvalves for microfluidic control, and a 7-cm-long CE separation channel. In addition, a new coinjector and sizing standard reservoir are integrated into the PCR-CE system to facilitate co-injection of a sizing ladder for STR size calibration. The portable genetic analyzer instrument has previously been described in detail. The instrument contains a 488-nm frequency doubled diode laser, a confocal fluorescence 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. A LabVIEW graphical interface (National Instruments, Austin, TX) was used to control the system through two DAQ (Data Acquisition) boards installed in a laptop.

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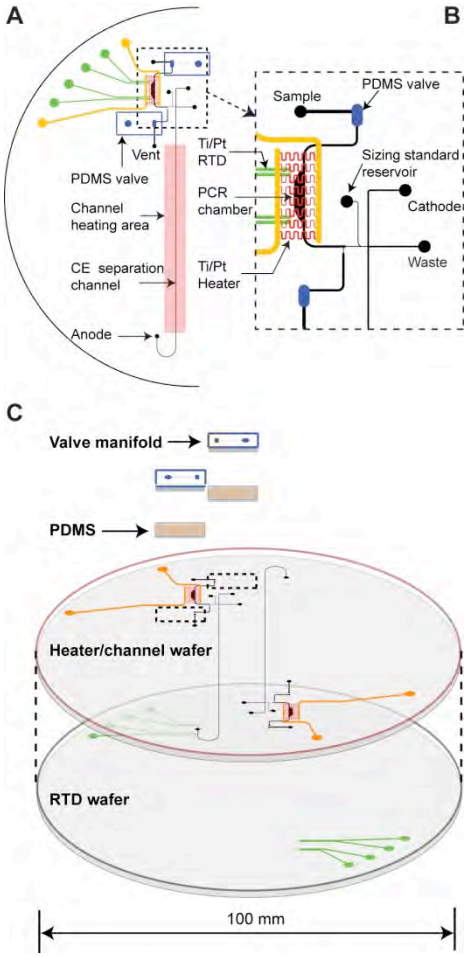

Figure 1. (A) Design of the PCR-CE microchip for forensic DNA analysis. The integrated device consists of 7-cm-long electrophoretic separation channels (black), 160-nL PCR chambers (black), RTDs (green), PCR heaters (red), and PDMS microvalves (blue). A coinjector, including a co-injection channel and a sizing standard reservoir is incorporated into the microdevice. (B) Expanded view of the heater, RTD, PCR chamber and CE co-injector. (C) Exploded view of the PCR-CE microchip assembly showing the valve manifold that controls the PDMS membrane valves, the PCR heaters fabricated on the upper surface of the heater/channel wafer, the glass microchannels etched in the lower surface, and the RTDs on the top of the lower wafer.

Sample preparation and chip operation. For proof of concept, we constructed a 9-plex autosomal STR typing system using primer sequences employed in PowerPlex® 16. Based on the primer concentrations provided by Promega, the 9-plex system was iteratively optimized to ensure balanced STR profiles. 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. Two standard commercial genomic DNA controls, 9947A female and 9948 male DNA (Promega, Madison, WI), were employed in the standard DNA test and limit-of-detection analysis. The 10-µL PCR mixture prepared for each experiment is comprised of 1.5× 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), DNA templates at the empirically dervived amount, the primer mixture, 6 U of FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN), and deionized water.

The microchip is prepared for PCR-CE analysis following the same protocol as described previously. The separation matrix, 5% (w/v) linear polyacrylamide (LPA) with

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6 M urea in 1×Tris TAPS EDTA (TTE) buffer, is loaded into the CE separation channel and the PCR cocktail is pumped in the PCR reactor. The modified thermal cycling protocol starts with an initial activation of the Taq polymerase at 95 ºC for 4 min followed by an initial template denaturing at 96 ºC for 1 min. In the first 10 of 32 PCR cycles, the temperature is held at 94 ºC for 30 s denaturing, then ramped to 60 ºC at 0.5 ºC /s for 30 s annealing, and then to 70 ºC at 0.2 ºC /s for 30 s extension. In the next 22 cycles, all the PCR parameters are kept the same except for the denaturing temperature which is reduced to 90 ºC. Finally, a post extension step is performed at 70 ºC for 10 min. Total PCR time is about 2 hr.

Once the thermal cycling is complete, the denaturing CE separation is performed on a channel preheated to 70 ºC. The MegaBACE™ ET550-R sizing standard solution (15 μL, GE Healthcare, Piscataway, NJ) is pipetted into the sizing standard reservoir. The microvalve adjacent to the sample reservoir is opened, and the amplified sample and the sizing standard are electrophoretically injected towards the waste reservoir simultaneously by applying an electric field of ~100 V/cm on the PCR reactor channel and co-injection channel while floating the anode and cathode. A separation field of 250 V/cm is then applied between the cathode and anode for electrophoresis. At least three injections and separations are performed to confirm the results, and each separation only takes about 8 min. After each analysis, the glass manifolds are removed, the PDMS membrane is replaced, and channels and chambers are cleaned using a fresh piranha solution (7:3 H_2SO_4: H_2O_2) to prevent carryover between runs.

Allelic ladder separation. The separation capability of the portable PCR-CE system for the chosen multiplex was first explored. The CE separation of a preconcentrated PowerPlex® 16 allelic ladder (4×) and an ET-550 sizing ladder using this microdevice was completed in 8 min, which is approximately 20 min faster than the single-capillary ABI 310 instrument. As shown in Figure 2 Panel A, the TH01 9.3 and 10 alleles, which differ by only one base pair, were distinguished with a resolution of 0.45. Alleles which differ by 2 bp in D21S11 were also successfully separated. Although the D13S317 and D8S1179 loci show weaker peak intensities resulting in imbalanced profiles, all the alleles in the 9-plex STR system were successfully resolved and sized, demonstrating that our microsystem can separate DNA fragments in the 106-

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259 bp range for forensic human identification. These results were used to generate the bin information in the MegaBACE™ program for designating the alleles in the subsequent STR analyses. Higher separation resolution can be readily achieved by simply employing a longer separation channel in the microchip design at the expense of slower separations. However, we elected to use the 7-cm channels in this study because of our focus on rapid point-of-analysis operation.

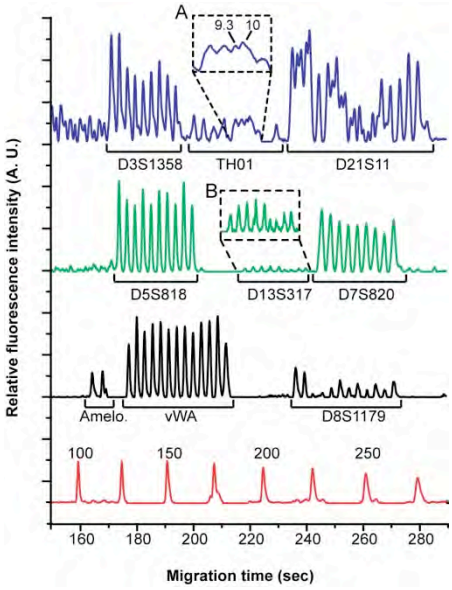

Figure 2. The 9-plex STR allelic ladder separation obtained on the portable PCR-CE microsystem. (A) The TH01 locus trace has been expanded to show the 9.3 and 10 alleles which exhibit a resolution of 0.45. (B) The D13S317 locus is similarly expanded for display. D3S1358, TH01, and D21S11 loci are labeled with FAM (first trace). D5S818, D13S317, and D7S820 are JOE (second trace). Amelogenin, vWA, and D8S1179 are TMR (third trace). The sizing standard (fourth trace) is MegaBACE™ ET550-R, labeled with the FAM-ROX energy-transfer dye.

Standard DNA test. The 9-plex autosomal STR amplification and separation on the portable PCR-CE microsystem was demonstrated using standard female 9947A and male 9948 genomic DNA. Figure 3 shows representative STR profiles of each sample amplified from 100 template copies in the PCR reactor. Each standard DNA template was analyzed in triplicate in independent amplifications and separations using the same conditions. All the alleles in each analysis were resolved, balanced, correctly sized and designated using the MegaBACE™ program.

In the co-injection structure used here, PCR amplicons and sizing standards are injected into the injection channel simultaneously. To evaluate the sizing calibration accuracy, the run-to-run standard deviations (n=3) of the allele sizes were calculated from the traces of 9947A and 9948 DNA obtained in the standard DNA test. As shown in Figure 3 C and D, all the alleles can be sized correctly with a standard deviation ≤ 0.8 bp. Although the D13S317 alleles in the 9947A DNA analyses show a higher deviation (0.8

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bp) due to their breadths, there is little injection mobility bias in the new injection structure demonstrating that our microsystem provides the necessary allele calling accuracy for human identification.

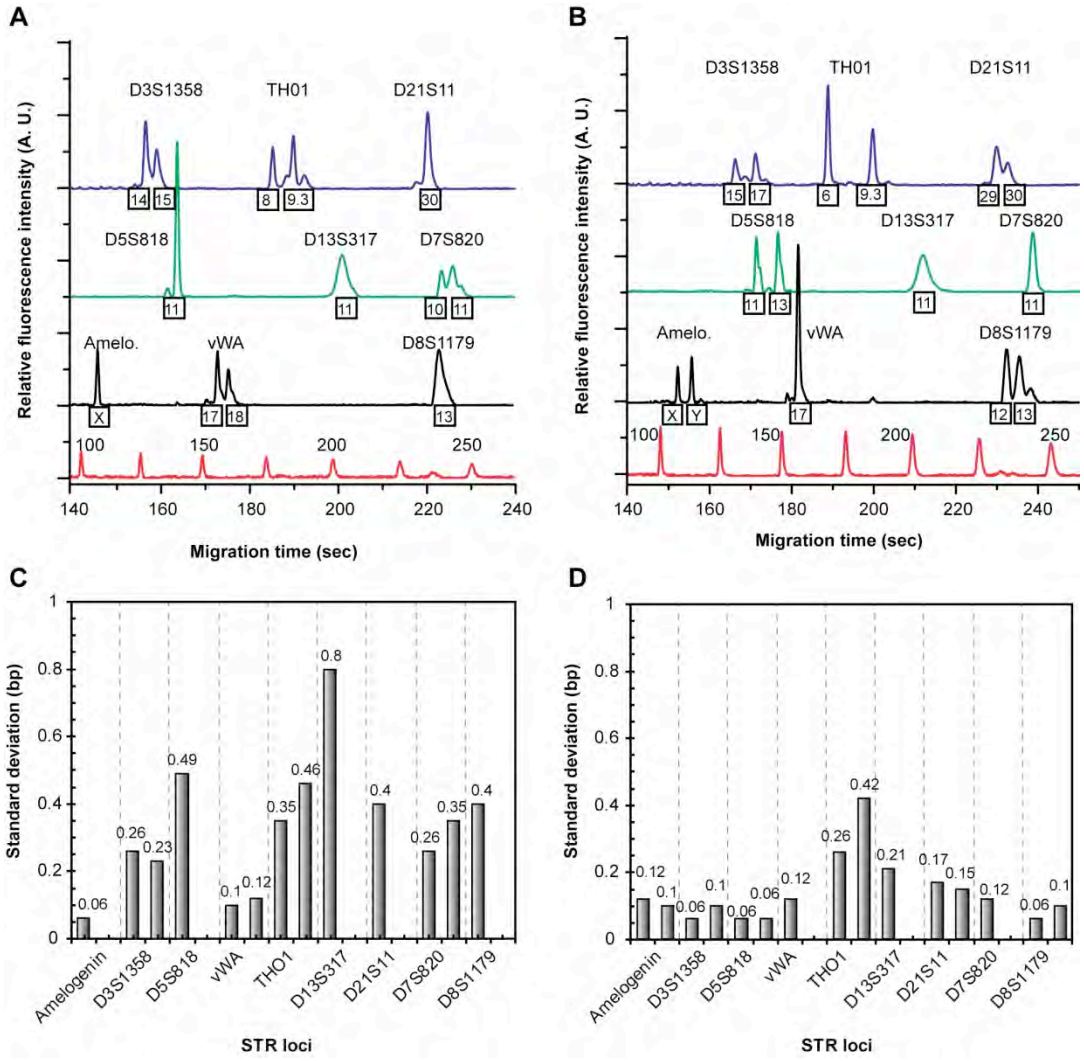

Figure 3. Representative 9-plex STR profiles of (A) 9947A female and (B) 9948 male standard DNA obtained with 100 copies of DNA template in the PCR chamber. The run-to-run standard deviations of the allele sizes for (C) 9947A and (D) 9948 DNA amplified and co-injected with sizing standards (ET550-R) using the PCR-CE microdevice on the portable instrument. Each sample was tested 3 times in independent amplifications and separations.

Limit of detection study. The sensitivity of the portable PCR-CE microsystem was evaluated using the 9-plex STR samples amplified from serially diluted 9947A standard DNA (200, 100, 50, 20, 10 copies of templates in the PCR chamber). Figure 4 shows the average percentages of full 9-plex STR profiles of 9947A DNA obtained from 3 runs at

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each DNA concentration as a function of input DNA template in the PCR reactor. Complete profiles (100% profile percentage) can be reproducibly obtained from 100 template copies. As the template concentration decreases (<100 pg or <33 copies), imbalance within two heterozygous alleles occurred, causing allele dropout. As shown in Fig. 4, when the DNA concentration was lowered to 50 copies, two full and one partial profile with one allele dropout were obtained, producing a 94.9% profile. With only 10 copies, 61.5% of the alleles are successfully amplified and detected. From the limit-of-detection study, the minimal template concentration needed to reliably produce complete DNA profiles was 100 template copies in the PCR reactor. We also found that excessive DNA concentration (>500 copies) results in split peaks or massive stutter peaks (data not shown).

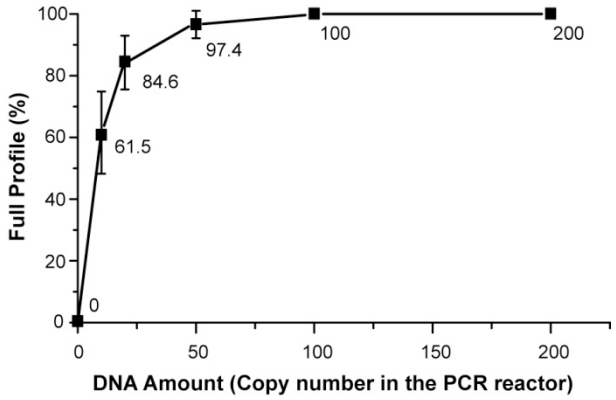

Figure 4. Percentage of full 9-plex STR profiles obtained from 9947A standard DNA on the portable microsystem as a function input DNA. With 100 template copies in the PCR reactor, full profiles are reproducibly obtained.

DNA analysis at a mock crime scene. To critically evaluate the capabilities of our portable microsystem and its compatibility with current crime scene investigation processes, real-time STR analyses were carried out at a mock crime scene prepared by the Palm Beach County Sheriff's Office (PBSO). The PBSO Forensic Biology Unit prepared blood stain samples on paper towels and cloth, representing a male and a female victim, and a male suspect, from known individuals with documented DNA profiles. The crime scene was set up in a pavilion at Lake Lytal Park, Palm Beach, FL by PBSO crime scene investigators. Two body-shape cardboard dummy victims were laid on the ground. The blue cloth shirt (male victim blood stain) was placed on top of a dummy, one of the paper towels (female victim blood stain) was placed next to the dummies, and the other paper towel (male suspect blood stain) was placed on top of a park bench nearby the

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scene (Fig. 5A and B). A PBSO Mobile Command Unit (MCU) was deployed to the mock crime scene, as shown in Fig. 5C. This MCU is equipped with a power generator, an air conditioning system, and a satellite internet connection, which are necessary for the on-site STR analysis. The DNA extraction equipment and materials were located in a separate room from the portable PCR-CE instrument to prevent interference between these two steps.

Figure 5. (A and B) Photographs of the mock crime scene investigated by PBSO in West Palm Beach, FL. Three blood stains on cloth or paper towels were laid out in the mock crime scene. (C) The entire DNA analysis was conducted in a mobile command unit provided by PBSO. (D) The portable forensic analysis instrument set up in the mobile command unit.

All DNA samples were extracted using the Maxwell 16® instrument with DNA IQ™ Casework Sample Kit (Promega) following the manufacturer's protocol. After the Maxwell run was completed in 30 min, DNA extracts in the final tubes were concentrated using Microcon columns to a final volume of 7 μL. Four microliters were used for the

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on-site analysis; the remaining sample and unprocessed blood stains were stored for possible future analyses. The PCR cocktail (4 μL of DNA sample in 10 μL of PCR cocktail) was loaded into the PCR reactor with a target concentration of 300 template copies in the 160-nL reactor. The PCR step was performed following the above described protocol (2 hr) and then the CE separations were performed 3 times (30 min). During thermal cycling, another microdevice for the next sample was prepared for streamlined operation.

The timeline of the field test is described as follows: We arrived in Lake Lytal Park at 7:00 am. The mock crime scene was set up and investigated by a crime scene investigator from PBSO. At 7: 30 am, DNA extraction was performed on the Maxwell system (Promega) and completed in two hours. At 9:30 am, the PCR cocktail containing the male suspect DNA sample (blood on a paper towel from the park bench) was prepared and loaded into the PCR-CE microdevice. The STR analysis on the portable instrument began at 10:00 am with the 2-hr PCR amplification and 30-min electrophoretic separation. At 12:30 pm, the male suspect profile was reviewed and submitted by email to PBSO to be searched in the local level CODIS database. A profile hit was generated and relayed back to the crime scene within twenty minutes. From sample collection to the generation of the CODIS hit, the entire process took only six hours. Between 1:00 pm to 6:00 pm, the blood stains from the male (shirt) and the female victim (paper towel) samples were also successfully typed on the portable instrument. Figure 6 presents the electropherograms of the three samples analyzed with the 9-plex STR system. These results demonstrate that the portable PCR-CE system can be deployed to a crime scene where it successfully performed on-site real-time STR typing without interference with the crime scene investigation process.

This work establishes the feasibility of using our portable PCR-CE microdevice for real-time forensic STR analysis at a crime scene or a security location. It presents some interesting topics for forensic policy and practice. The on-site human identification could be used for real-time analysis at crime scenes to facilitate case detectives' investigation before the suspect has fled the area or destroyed secondary evidence. Second, in states that require DNA samples at arrest, the ability to rapidly type an arrestee before release on bail might generate probable cause to hold the individual

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because of hits on other earlier criminal activities. In the current study, the use of 9-plex STR system is not limiting, although with further device and process improvements, extension to 16-plex is possible. Due to the limited throughput of the current portable microsystem, only three blood stain samples were analyzed consecutively, and no positive and negative controls were included in the field trial. We recognize that a multi-lane microdevice that can process 4-6 samples in parallel is desirable. In the future, a sample purification and concentration structure integrated with on-chip PCR and CE is desirable to facilitate STR analysis from real-world samples and to efficiently load DNA into the nanoliter PCR reactor. Furthermore, an affinity-capture-based in-line injector in place of the inefficient cross-injector would also enable more sensitive analysis.

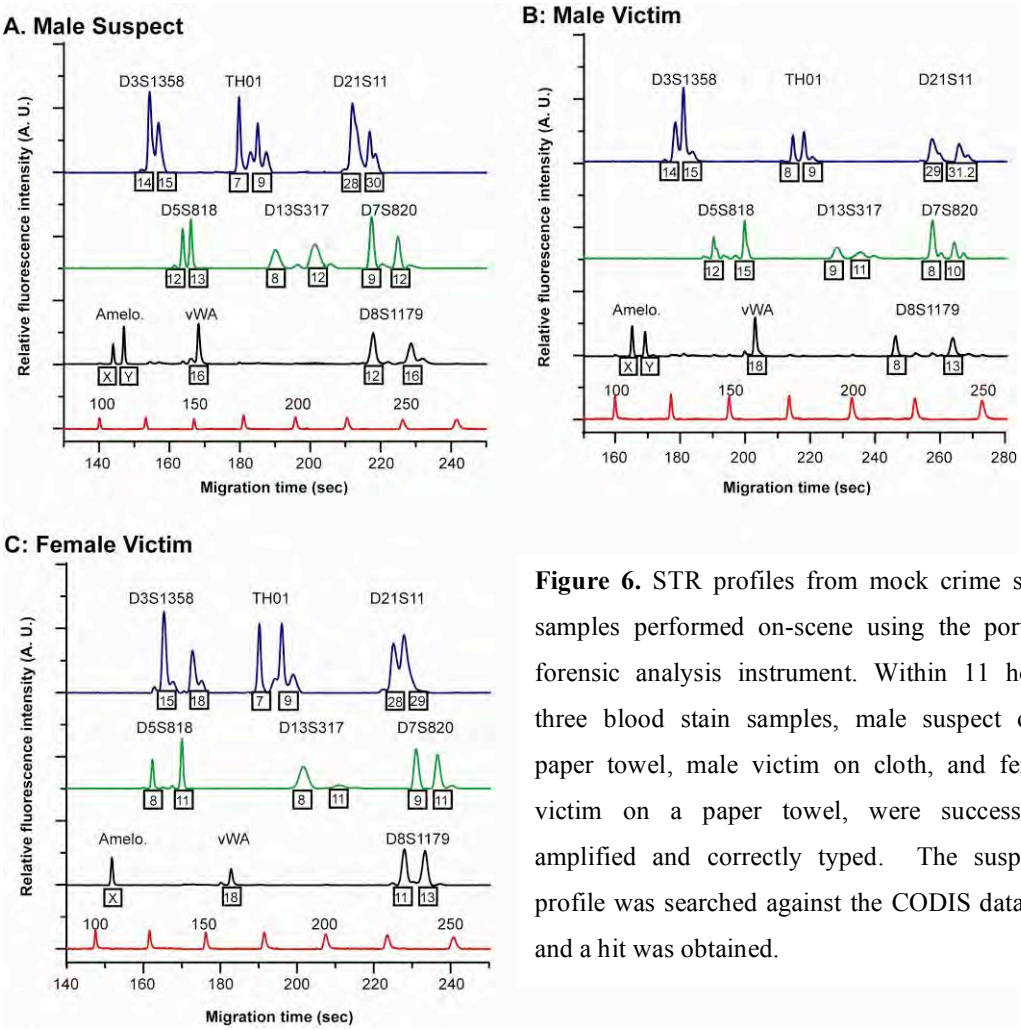

Figure 6. STR profiles from mock crime scene samples performed on-scene using the portable forensic analysis instrument. Within 11 hours, three blood stain samples, male suspect on a paper towel, male victim on cloth, and female victim on a paper towel, were successfully amplified and correctly typed. The suspect's profile was searched against the CODIS database and a hit was obtained.

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*2. Development of **Single-Channel** Sample Cleanup and CE Device*

Current STR analysis by CE bypasses the post-PCR sample cleanup step in order to save time and cost. However, the injection from high-salt PCR samples exhibits poor injection efficiency and is biased against larger loci. Additionally, the cross-injector geometry employed in the conventional CE microchips requires delicate timing and electric field balance. To address these issues, we have developed an integrated STR sample cleanup, concentration method that employs a photopolymerized streptavidin-gel capture chemistry (Figure 7A) coupled to a simple, improved direct injector geometry (Figure 7B & C) to achieve higher fluorescence signals and sensitivity for DNA typing. Results of this work and the technical details were disseminated in *Analytical Chemistry*.[46]

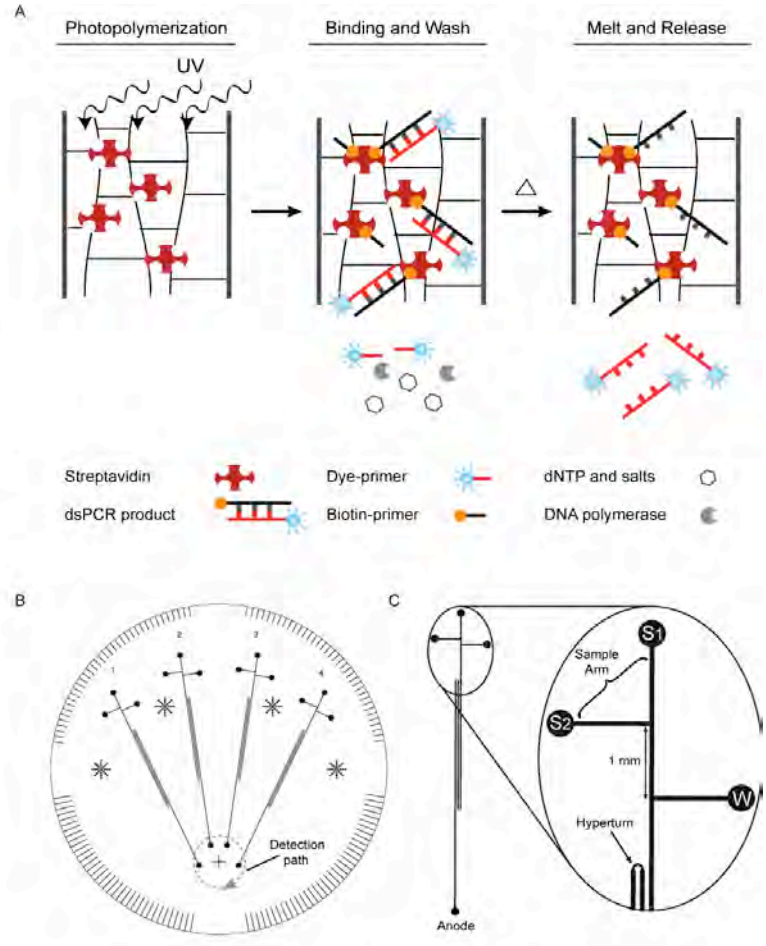

Figure 7. (A) Schematic of the streptavidin-gel capture method for purifying and concentrating biotin-modified PCR products. One primer labeled with fluorescent dye and one with biotin are used to generate dsPCR products. The PCR reaction is electrophoresed through the cross-linked polyacrylamide get network

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where the ds-DNA products bind strongly to the streptavidin gel. Unreacted PCR materials are washed off and then the fluorescently labeled DNA strand is thermally released from its complementary captured strand for electrophoresis down the separation column. (B) Design of the 4-channel integrated capture-μCE array. This 100-mm diameter array contains four individual processors each of which contains a 1-mm offset in which a capture gel is created by photochemistry using riboflavin as a photoinitiator. The capture DNA plug travels down a folded 10-cm separation column and is detected by laser induced fluorescence (LIF).

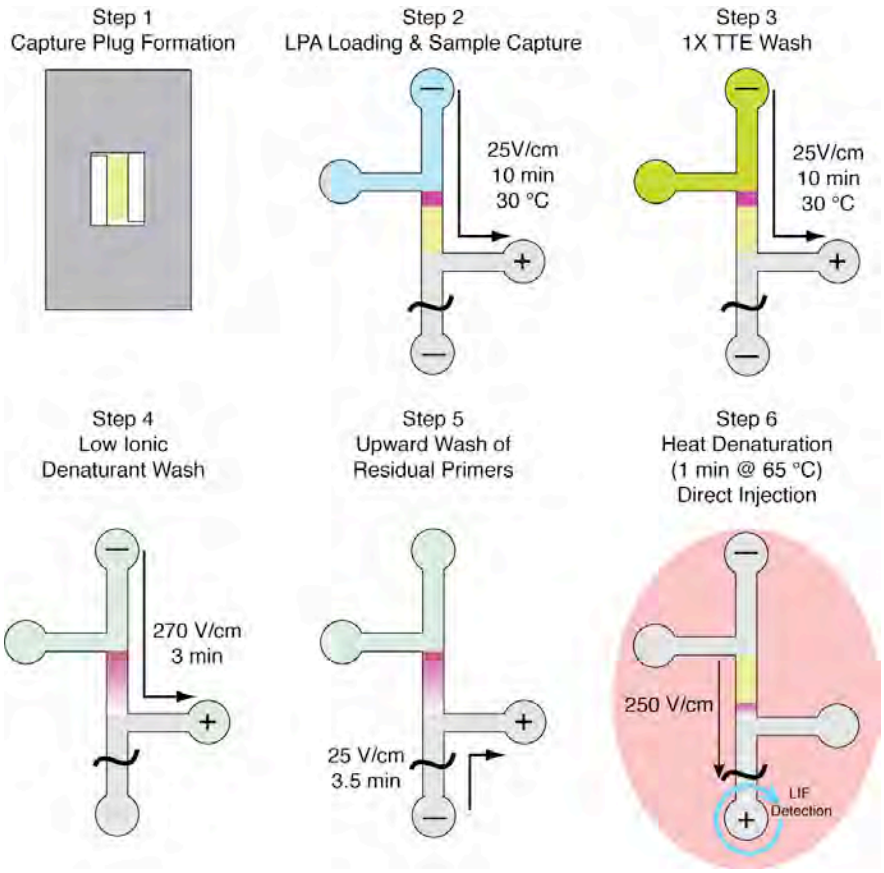

Figure 8. Schematic of the integrated streptavidin capture-CE process. (Step 1) A photopolymer solution containing streptavidin/bis-acrylamide is loaded in the microchannel and exposed to UV radiation in the capture zone though a photomask to created a ~ 1-mm capture gel plug. (Step 2) Separation gel is loaded from the anode up the microchannel to the waste reservoir. Amplified PCR sample is then loaded into the sample arm and an electric field of 25 V/cm is applied between the sample and the waste at 30 °C to initiate the capture process. A small voltage is applied to the anode at the same time to keep any unbound materials from entering the separation channel. (Step 3) The captured DNA products are then washed with fresh 1X TTE buffer under the same conditions. (Step 4) A deionized formamide solution is passed through the captured sample to achieve stacking and to lower the release temperature during electrophoresis. (Step 5) An upward wash with 1X TTE buffer is carried out to wash any residual unbound materials towards the waste. (Step 6) The region above the capture plug is replaced with 1X TTE and the microdevice is

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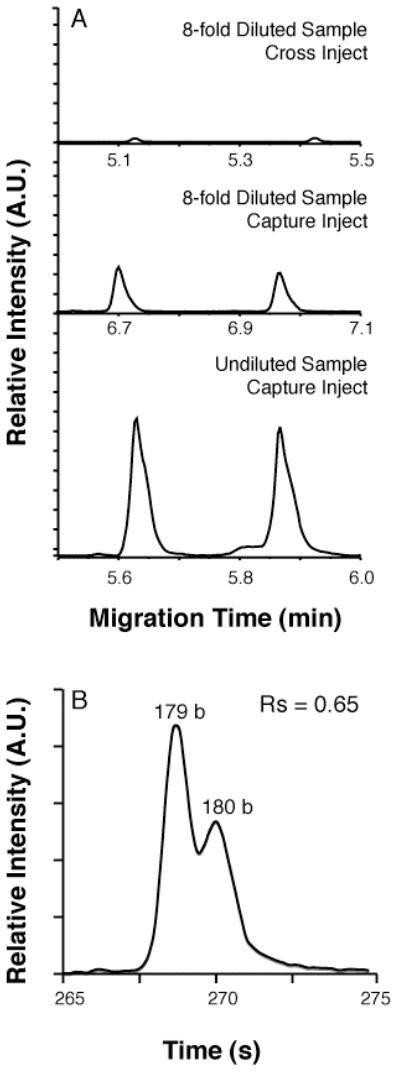
equilibrated at 65 °C for ~ 1 minute to thermally release the fluorescently labeled DNA strands, which are electrophoresed at 250 V/cm towards the anode and interrogated by the Berkeley 4-color rotary confocal scanner.

As illustrated in Figure 8A, a streptavidin-capture gel zone is created and immobilized by photochemistry. Biotin-labeled double-strand PCR products generated using one biotin and one fluorescence-labeled primer are electrophoresed through the capture gel bed where they are bound efficiently via biotin-streptavidin interaction. Unbound materials are washed off and the biotin-dsDNA products are retained in the capture gel for subsequent thermal release for electrophoresis. The purification and CE operation steps shown schematically in Figure 8 take only ~ 40 minutes.



The following summarizes results from our evaluation of fluorescence intensity enhancement and resolution, STR typing capability, detection limit and typing of simulated degraded DNA samples using our new integrated sample processing method and chip.

Figure 9. (A) Electropherograms of a biotin-modified monoplex PCR sample amplified from 0.5 ng of 9948 DNA. The top trace was obtained by cross injection of an 8-fold diluted PCR sample. The middle trace was obtained by the affinity capture-μCE device for the same diluted sample. The bottom trace was obtained by the affinity capture-μCE device with the undiluted PCR reaction. (B) Electropherogram obtained on the affinity capture-μCE device for a PCR sample containing DNA fragments 179 and 180 bases in length, demonstrating a resolution of 0.65.

Integrated capture-μCE enhances fluorescence intensity. For a diluted sample, the fluorescence intensity of the TH01 alleles shown in Figure 9A is enhanced by at least 10 fold compared to cross injection using this streptavidin capture–μCE device and method. When an undiluted PCR sample is used, the signal intensity is

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enhanced by ~ 50 fold. Also, DNA fragments that differ by a single base can be differentiated with an average resolution (Rs) of 0.65 (σ = 0.1) and a valley value of (Vv) of 50% (σ = 3%) as shown in Figure 9B. This resolution is comparable to that obtained for high-quality DNA genotyping obtained on both microchip and commercial CE platforms,[27, 47] demonstrating that the inline affinity capture produced a high-quality CE injection.

Integrated streptavidin–μCE analysis of STR samples. To evaluate this capture-μCE device for STR typing, a biotin-modified 9-plex STR typing kit has been constructed and balanced using primer sequences and fluorescent dye-labels found in the PowerPlex® 16 kit. The STR loci included in the 9-plex system are amelogenin for sex typing, vWA, D8S1179, D3S1358, TH01, D21S11, D5S818, D13S317 and D7S820. PCR samples amplified using these house-made kits were analyzed and compared to separations obtained with cross injection.

As shown in Figure 10, a 14-fold increased fluorescence intensity was observed for the diluted 9-plex reactions amplified from 0.5 ng of standard human genomic DNA using the streptavidin-μCE analyses compared to separations with the cross-injector device under the same detection settings. Similar peak balance across the DNA profile was maintained for both analysis methods, and was observed for a variety of DNA quantities and samples (data not shown). When pure amplified PCR samples were used, the fluorescence intensity was increased by ~19X. The excess primers and the TMR dye peak observed in the cross-injection sample were also completely removed by the purification process. The fluorescence intensities of the D13S and D8S allele peaks in the undiluted sample exceeded the range of out analog/digital converter, producing the split-peak appearances after color-crosstalk correction. The signal strength of the multiplex samples was not enhanced to the same magnitude as the monoplex samples simply due to the higher number of alleles of different sizes that have to be captured by the same amount of streptavidin in the gel. These results demonstrate the capability of the streptavidin capture method to purify and concentrate simple and high-order multiplex PCR reactions to achieve higher fluorescent signal intensities and to eliminate dye label interference. The complete removal of primer peaks will be especially beneficial for miniSTR analyses [21] by opening up space in the electropherogram for smaller molecular

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weight marker sets.

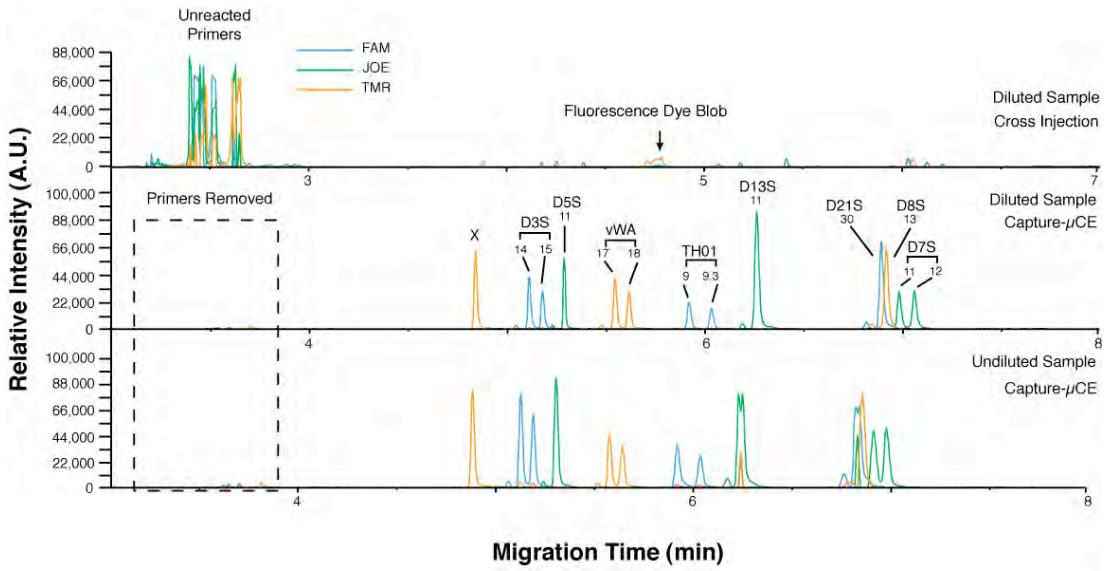

Figure 10. DNA profiles amplified using the biotin-modified 9-plex kit from 0.5 ng of 9947A DNA. The top trace was obtained by cross injection of a 8-fold diluted PCR sample. Intense primer peaks dominate the electropherogram and a TMR dye blob overlaps with the vWA alleles. The middle trace was obtained using the capture-μCE device for the same diluted PCR sample. The bottom trace was obtained with the capture-μCE device for the undiluted PCR sample.

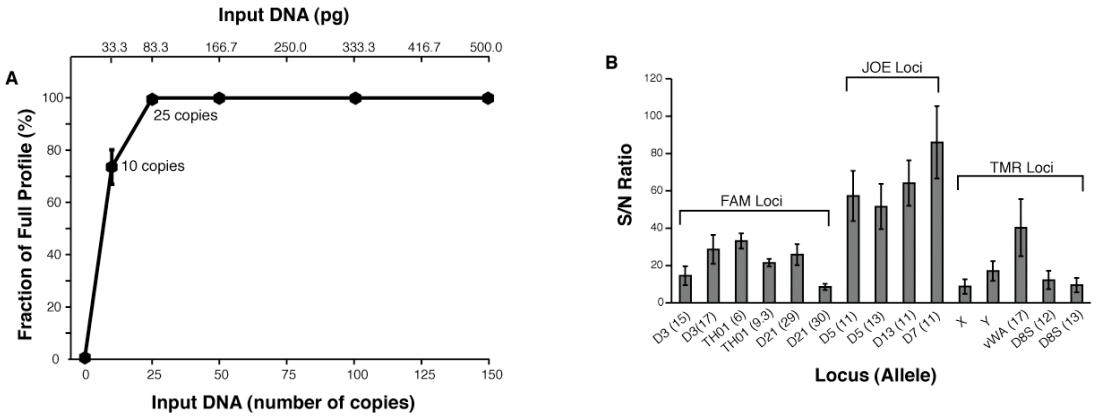

Figure 11. (A) Percentage of full STR profiles obtained using the capture–μCE device for 9948 DNA samples amplified with the biotin 9-plex as a function of input DNA copy number. Full STR profiles were reliably obtained from as low 25 copies of DNA template. (B) Average allele signal-to-noise (S/N) ratios for the 25-copy DNA sample on the capture-μCE device. The homozygous D13, D7 and vWA loci produced single allele peaks about twice the heights of the heterozygous allele peaks.

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Limit of Detection. A sensitivity study was carried out to evaluate the detection limit of the streptavidin–μCE method by using biotin-modified 9-plex PCR reactions amplified from serially diluted 9948 standard human genomic DNA. Three independent tests were performed at each sample concentration. Figure 11A plots the percent allele detection as a function of DNA template copy number. All 15 expected STR alleles were successfully and reproducibly detected (defined as S/N ≥ 3) with as few as 25 copies of DNA input. The average S/N ratios of each allele at the 9 loci for the 25-copy sample are shown in Figure 11B. This is half the input DNA typically required for forensic multiplex typing of a similar number of STR loci on commercial CE instruments and for cross-injection based microchip platforms. Stochastic effects were also observed for the 25-copy samples. However, the enhanced fluorescence intensities achieved using the capture–μCE device should make the minor alleles more readily detectable in situations where one of the sister alleles has a disproportionately smaller peak height.

Degraded DNA typing. Four simulated degraded DNA samples were prepared and provided by the VDFS to test the ability of the streptavidin capture–μCE method to improve analyses of compromised DNA samples. Blood samples collected from two individuals were each exposed to 56 and 80 °C for 3 months and stored for six years to simulate degradation. The extracted DNA samples were amplified using the 9-plex biotin system at two DNA input amounts (Table 1). Only one amplification was prepared for sample B1 due to DNA scarcity. Since our microchip-based CE system provides similar performance to that of conventional CE systems in terms of sensitivity, each amplified sample was processed by the capture–μCE device and compared to results obtained using the cross injection. The obtained profiles were assessed using the standard allele calling procedure developed previously in our lab.[26] DNA profiles generated with the capture–μCE device have overall higher fluorescence intensity and a complete absence of primer interference and dye blobs. More alleles were detected with the capture–μCE device than the corresponding profiles obtained using cross injection as summarized in Table 1. Three full DNA profiles were obtained using the capture–μCE device while none of the profiles obtained using cross injection yielded all the expected alleles. These full profiles displayed 4 – 8X higher fluorescence intensities for the larger alleles. These larger alleles were below the limit of detection in profiles obtained with cross injection.

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For DNA samples that resulted in no alleles (Fig. 12A) or a few alleles (Fig. 12B) with cross injection, ~ 33% and ~71% more allelic markers were obtained, respectively, using the streptavidin capture–μCE method. While preferential amplifications of the smaller DNA fragments and drop-out of the larger alleles were still observed for these low-integrity samples, more of the higher molecular weight fragments were detected because of the higher fluorescence signals associated with the affinity-capture injection.

Table 1. Simulated degraded DNA samples

DNA Sample	Degradation Conditions (°C)	DNA Extract (ng/ μL)	Input DNA (μL)	Input DNA Amount (ng)	Cross Injection Percent Profile (%)	Capture-μCE Percent Profile (%)
A1	56	0.14	5.0	0.700	46.7	100.0
			2.5	0.350	0.0	20.0
A2	80	0.00975	5.0	0.049	0.0	20.0
			2.5	0.024	0.0	6.7
B1	56	0.114	5.0	0.570	82.4	100.0
B2	80	0.261	5.0	1.305	82.4	100.0
			2.5	0.653	11.8	70.6

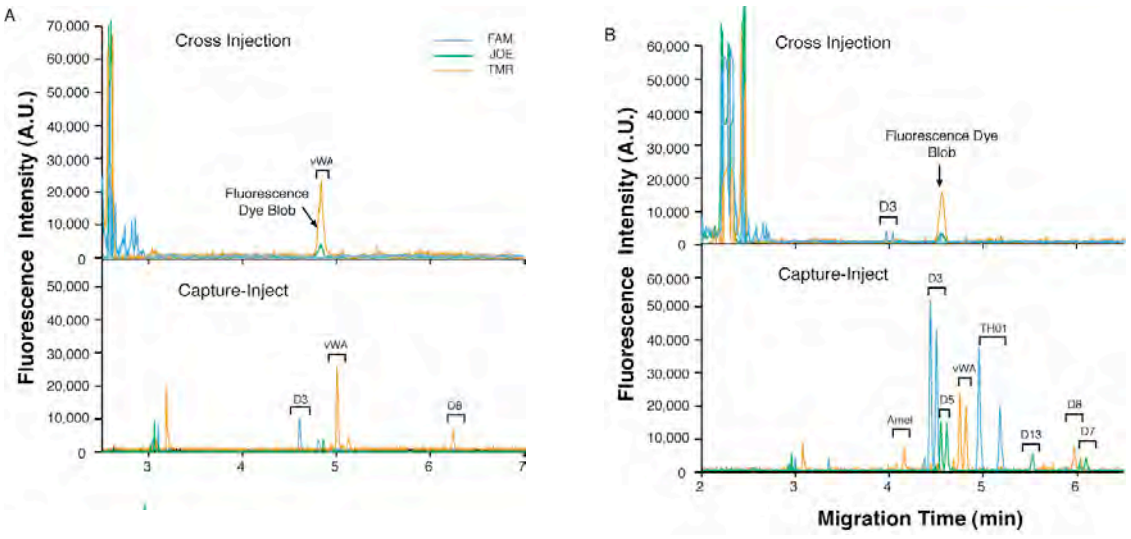

Figure 12. Analyses of PCR reactions amplified from two degraded DNA samples. (A) For sample A2, no alleles were detected using the cross injection method and a dye blob is observed overlapping with a putative vWA peak. Analysis of the same sample with the capture-μCE device and method produced three alleles at D3, vWA and D8 loci with fluorescent counts of 10K, 30K and 5K counts. (B) For sample B2, two D3 alleles were detected using the cross injection method and a dye blob observed overlapping with

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the putative vWA alleles. Analysis of the same sample with the capture–μCE process yielded ~ 10X higher fluorescent intensity for the two D3 alleles and 10 additional alleles with signals from 5K to 30K fluorescent counts while eliminating dye blobs and primer signals.

The development of the photopolymerizable streptavidin-gel capture method coupled with a simple injector geometry for rapid purification and electrophoretic separation of STR amplicons allows the detection of STR alleles from half the input DNA normally required for electrokinetic injection of impure samples. This enhanced sensitivity enabled the capture of additional allele data from degraded DNA samples. The ability to precisely define the size and position of the capture gel can help improve forensic lab efficiency by dramatically increasing the fluorescence signals for low-level DNA samples, or by normalizing the DNA signals when the capture gel is a limiting reagent. This inline capture injection can be integrated with our multi-lane μCAE or on-chip PCR method, leading to the realization of high-throughput, and high-performance genetic analysis systems for a variety of applications.

*3. Development of **multi-lane** Capture-CAE microsystem*

We have developed an integrated STR sample cleanup and separation microdevice for high-sensitivity STR analysis.[46] However, to make this device and method practically useful and cost-effective for routine forensic work, the scaling of this method to high-density array structures is essential. The inline capture injector can be incorporated into our previous microfabricated capillary array electrophoresis (μCAE) microsystem[2] to achieve the capability of high-throughput STR sample analysis. As shown in Figure 13, we designed a 12-lane capture-CAE chip using the same chip layout as those of our previous μCAE devices. On a 4" glass wafer, twelve 10-cm-long separation channels sharing a common anode are grouped into six doublets, each of which includes two capture gel inline injectors with two sample wells and one shared cathode and one waste well. The capture gel inline injector is a 500-μm-long double-T channel junction with a tapered structure for PCR product cleanup, concentration, and inline injection. The tapered structure is designed to keep the capture gel in place during gel loading. All features were isotropically etched to a depth of 40 μm and a final width of 160 μm using the same wet etching method as described previously.[26] This microfabrication process is simple and reliable. To achieve maximal sensitivity and to

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minimize manual operations, the capture and separation process, as shown in Figure 14, was carefully optimized using an imaging station. This study is a significant step towards the practical application of this integrated capture-separation process in forensic investigation. The manuscript of this work has been submitted to *Forensic Science International: Genetics.*

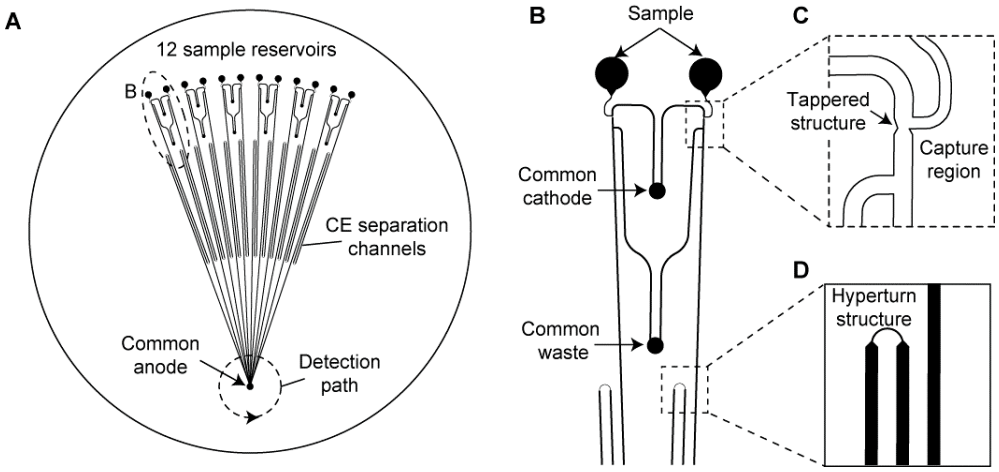

Figure 13. The schematic of the 12-lane capture-CAE microdevice. (A) A total of 12 electrophoretic separation channels coupled with capture gel inline injectors arranged on a 4" glass wafer, forming six doublets. (B) Each doublet includes two capture gel inline injectors and two sample wells sharing one cathode and one waste well. (C) The expanded view of the capture region. A tapered structure was designed in the top of the capture region. (D) The expanded view of the hyper-turn structure.

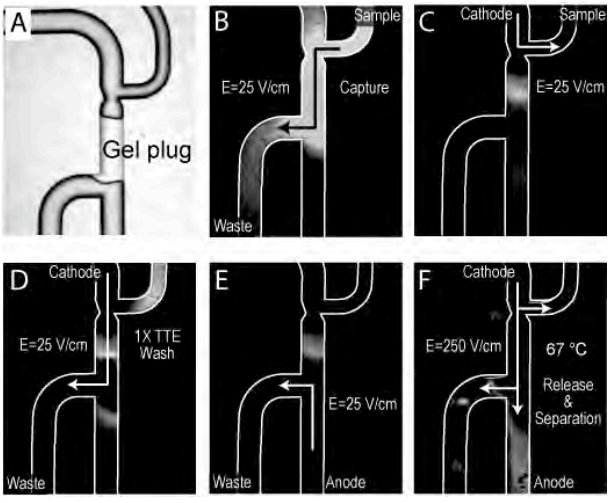

Figure 14. The capture-CAE operation process observed using an imaging station. (A) A ~500-μm capture gel plug containing streptavidin is formed in the capture channel by photopolymerization. Separation matrix is loaded from the anode to the waste and from the cathode to the sample. (B) Off-chip amplified PCR sample is loaded into sample well and injected to pass through the capture bed under an electric field of 25 V/cm at room temperature for 10 min. (C) After capture, an electric field of 25 V/cm is applied from the cathode to the sample reservoir to electrophorese the excess sample back. (D) The captured DNA products are then washed under the same electric field for 5 min to push all the uncaptured sample contents to the waste

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reservoir. (E) A backwash step is carried out to wash any unbound materials in the separation channel towards the waste. (F) The microdevice is equilibrated at 67 °C for ~ 1 minute to thermally release the fluorescently labeled DNA strands, which are electrophoresed at 250 V/cm towards the anode.

The following summarizes results from our evaluation of the capture-μCAE microdevice, including the capability of high-throughput STR typing, comparison with conventional cross injection, limit of detection, and touch evidence analysis.

High-throughput STR analysis. We analyzed 9-plex STR samples amplified from 50 copies of 9947A standard genomic DNA on the 12-lane microdevice to test the chip design and the operation protocol for high-throughput forensic STR typing. As shown in Figure 15, STR profiles were successfully obtained from all the 12 lanes in 30 min. The average percentage standard deviation of the allele signal-to-noise (S/N) ratios is 18.8 % and the average standard deviation of the allele migration time is 9.8 sec. These variations are similar to those in conventional chip-based μCAE separations, and can be effectively corrected by incorporating sizing standards. These results demonstrate that the current chip design and operation protocol are compatible with high-throughput integrated STR sample cleanup and analysis.

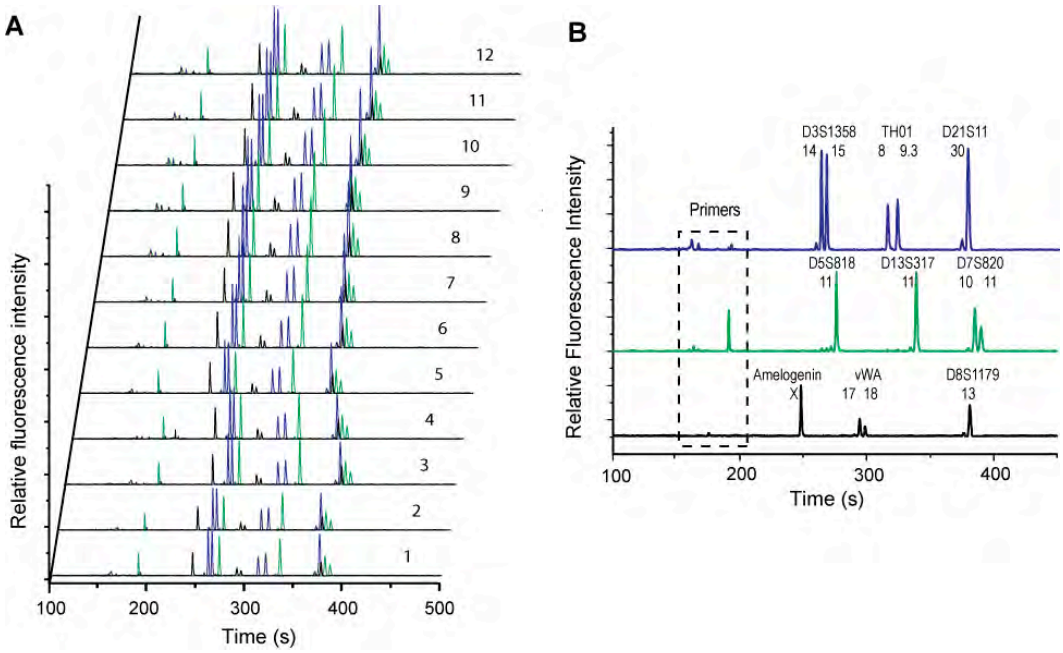

Figure 15. (A) Electropherograms from 12 lanes in a single run. All the traces are plotted in the same signal intensity scale demonstrating that all 12 channels can provide similar performance. (B) Example of one of the typical 9-plex STR traces obtained from the 12-lane μCapture-CAE microsystem. The dramatically reduced primer peaks show the effectiveness of our sample cleanup procedure.

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Comparison with conventional cross injection. Since the design of the 12-lane capture-μCAE device is similar to our μCAE system, both the capture inline injection and the conventional cross injection can be performed on the same capture-μCAE microchip, which allows us to directly compare these two methods to evaluate the sensitivity improvements. In this study, the same STR samples amplified from 50 copies of 9948 standard genomic DNA were analyzed by using both methods on the same microchip under the same detection settings. As shown in Figure 16, the allele S/N ratios in the 9-plex STR profile obtained using the capture inline injection was improved 12.1±1.8 fold over the cross injection. Similar peak balance across the DNA profile was maintained for both analysis methods. The excess primers observed in the cross-injection sample were completely removed by the purification process, demonstrating the effectiveness of sample cleanup, concentration and inline injection.

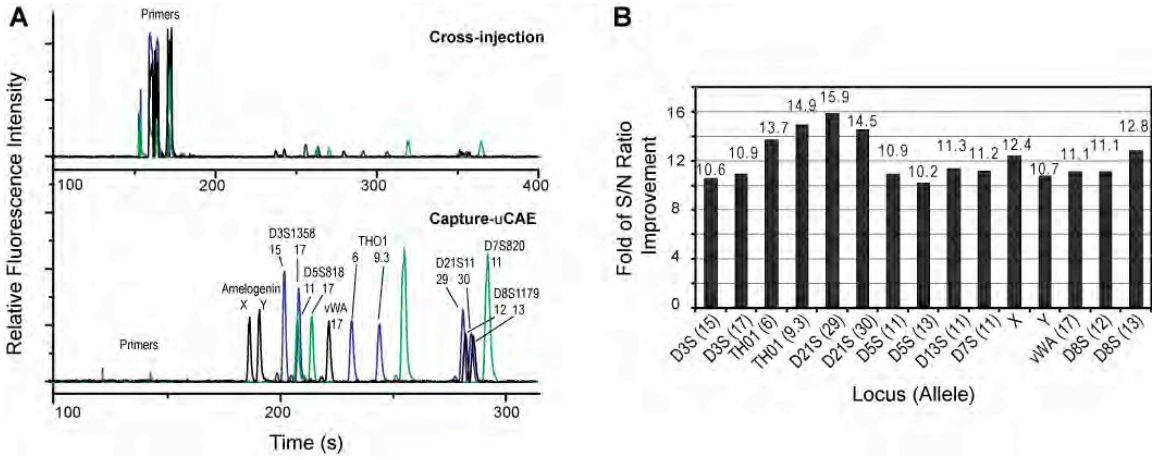

Figure 16. DNA profiles amplified using the 9-plex STR typing system from 0.15 ng of 9948 standard DNA. (A) The top trace was obtained by cross injection using the capture-μCAE device. The bottom trace was obtained using the same device with capture inline injection procedure. (B) Graph of the S/N ratio improvement on each allele. The average improvement by using the capture inline injection over the cross injection is 12.1±1.8 fold.

Limit of detection test. A sensitivity study was carried out to evaluate the detection limit of the capture-μCAE microsystem by using 9-plex STR samples amplified from serially diluted 9947A standard genomic DNA. Figure 17A plots the percent allele detection as a function of DNA template copy number. All 13 expected STR alleles were

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successfully and reproducibly detected with as few as 35 copies of DNA input. The average S/N ratios of each allele at the 9 loci for the 35-copy sample are shown in Figure 17B. This sensitivity is slightly lower than the previous single-lane system (25 copies) due to a change of the sample loading method. Since the samples are only loaded into the sample reservoirs instead of the entire injection channels, the injection into the capture region is less efficient. Nevertheless, the limit of detection is still improved significantly compared to commercial CE instruments and cross-injection based microchip platforms (≥ 50 copies).[26]

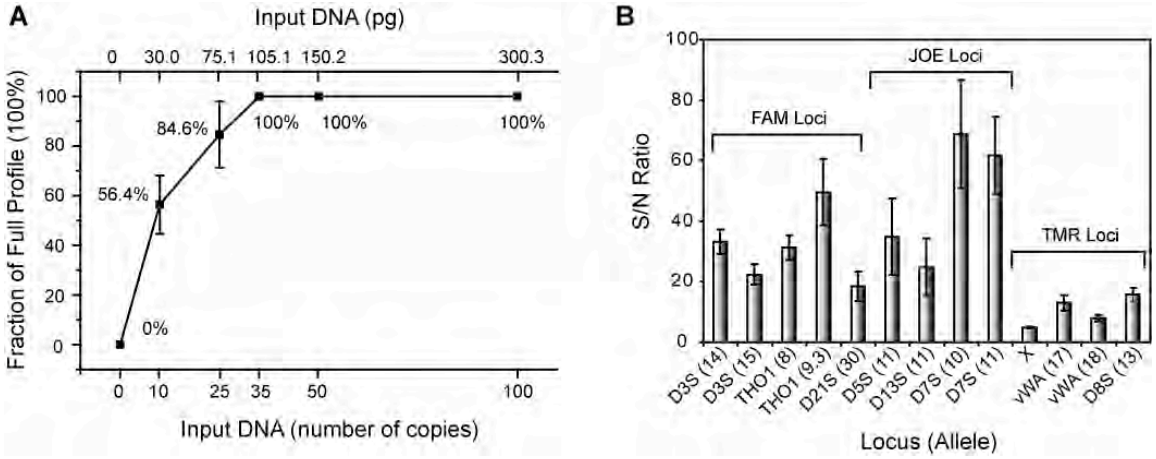

Figure 17. The limit-of-detection test of the capture-μCAE microsystem. (A) Percentage of full STR profiles obtained using the capture-μCAE device for 9947A standard DNA samples as a function of input DNA copy number. Full STR profiles were reliably obtained from as low as 35 copies of DNA template. (B) Average allele S/N ratios for the 35-copy DNA sample on the capture-μCAE device.

Touch evidence analysis. To test the capability of the capture-CAE system for low-copy-number DNA analysis, we analyzed touch evidence provided by the Virginia Department of Forensic Science. The touch evidence samples recovered from unfired bullet cartridges were prepared by the Virginia Department of Forensic Science (VDSF) following the procedure described previously.[48] Bullet cartridges were picked up out of box and loaded into a pistol by a volunteer with no additional or excessive handling of the bullet cartridges ("real conditions"). This pistol was then submerged in water for one hour. After collection from the submerged weapon, each cartridge was swabbed using the double swab technique.[49] DNA samples were extracted following the VDSF

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BioMek® 2000 Automation Workstation Procedures Manual for Large volume samples. DNA quantitation was performed using the Plexor HY System (Promega) on a Stratagene Mx3005PTM Real-Time PCR System (Cedar Creek, TX) according to the manufacturer's specifications with a recalibration of standard DNA concentrations. The samples were shipped to Berkeley for analysis on the capture-CAE device. PCR amplifications were performed with 4 µL input DNA in 12.5 µL reaction volume using the standard protocol.

In total, three samples, the concentrations of which are characterized as 4.1, 4.6, and 4.8 pg/µL, were tested on the capture-µCAE system. As demonstrated in Figure 18, 53%, 71%, and 59% of the 9-plex STR profiles were successfully obtained by using the sample cleanup, concentration and analysis method on the capture-CAE device. With the aid of biotin-labeled sizing standards, these alleles were successfully recognized and called for their repeat numbers. Pronounced up-stutter peaks were observed in this study due to the LCN amplification. As a comparison, the same samples were analyzed using the cross injection method under the same experiment settings, revealing consistent blank profiles. The DNA yields of the unfired cartridges from the control, un-submerged pistol were approximately 3 times that of the cartridges retrieved from the submerged pistol (17.9±22.6 pg and 5.9±8.1 pg, respectively; n=15), demonstrating the extremely challenging nature of these samples (unpublished data, S. Greenspoon). This study dramatically validates the advantages of the capture-CAE system for the analyses of "touch" or low-copy-number/low template DNA analysis.

The capture-CAE microsystem provides a reliable and robust platform for forensic STR typing of LCN and degraded DNA due to its seamless integration of analytical steps, automated operation process, and 100% efficient sample analysis. This system has the potential to become a routine way to reliably analyze DNA samples in forensic investigations. In the future, the development of UV exposure systems for rapid photopolymerization at multiple spots would enable a 96-lane capture-CAE device without major modification. The extension to 16-plex STR typing system is also possible without any significant obstacles on chemistry and operation. Additionally, this inline capture injection process can be incorporated into the on-chip PCR-CE system, leading to the realization of a highly sensitive, fully integrated system.

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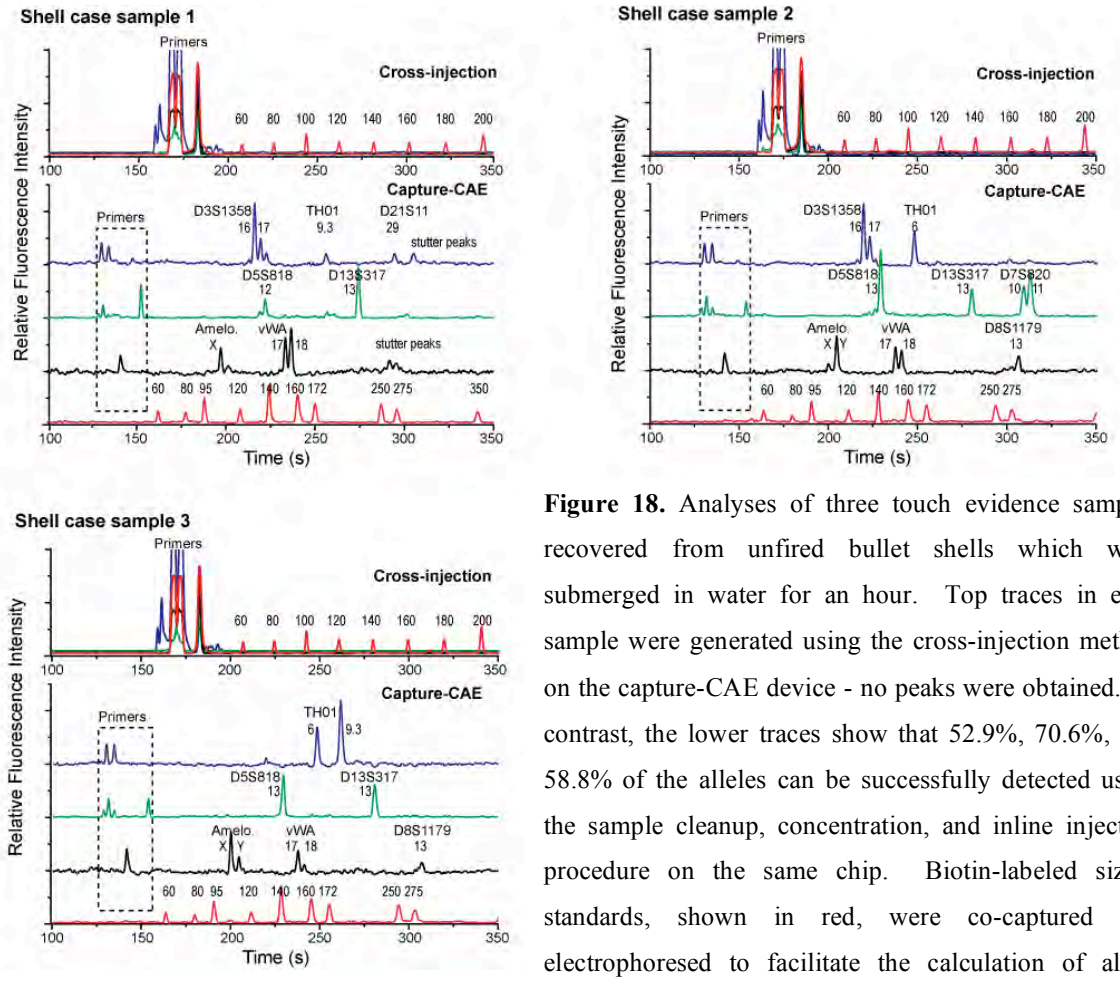

Figure 18. Analyses of three touch evidence samples recovered from unfired bullet shells which were submerged in water for an hour. Top traces in each sample were generated using the cross-injection method on the capture-CAE device - no peaks were obtained. In contrast, the lower traces show that 52.9%, 70.6%, and 58.8% of the alleles can be successfully detected using the sample cleanup, concentration, and inline injection procedure on the same chip. Biotin-labeled sizing standards, shown in red, were co-captured and electrophoresed to facilitate the calculation of allele repeat numbers.

4. Development of integrated template capture chemistries and methods

We have developed an integrated PCR-CE microdevice as well as a portable detection instrument for on-site rapid human identification. Real-time DNA analyses at a mock crime scene have been successfully conducted in collaboration with law enforcement agencies. While this demonstration validates the feasibility of STR typing using integrated devices, this microsystem falls short in its ability to analyze real-world samples containing PCR inhibitors; thus, integrating a DNA purification technique prior to the analysis is necessary. To address this issue, we successfully demonstrated the integration of the novel sequence-specific DNA extraction and the improved post-PCR capture inline injection into the well-characterized PCR-CE system to form a fully integrated microdevice for forensic STR analysis. Using the bead capture structure developed previously,[50, 51] a fluidized bed of streptavidin-coated magnetic beads captures

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the conjugates of biotin-labeled oligonucleotide probes and genomic DNA fragments containing the STR locus sequences. After DNA capture, the bead-DNA conjugates are pumped to a PCR reactor for 9-plex STR amplification. The resulting biotin-labeled PCR products are electrophoretically driven through a streptavidin-modified capture gel where they are bound and concentrated into a narrow injection plug, followed by thermal release for CE separation. By integrating these components on a single chip, we automate the process, improve the reliability, and minimize the risk of contamination during the sample analysis. This structure has enabled the successful STR typing from forensic samples in a fully integrated microdevice. A manuscript describing this work is in preparation.

Microdevice design. The microdevice shown in Figure 19 contains two identical genetic analysis systems forming a symmetrical doublet on a 4-inch glass wafer. The structure is similar to the device developed in our group previously,[51] but the design is modified to integrate the post-PCR cleanup and inline injection functions and to adapt to the newly developed scanner instrument. Each analytical system includes a PDMS micropump and two PDMS microvalves[41] for fluidic control, a 4-cm-long bead capture structure with a system of bifurcating channels for DNA template capture,[50] a 250-nL PCR chamber with a microfabricated heater and a resistance temperature detector (RTD) for PCR thermal cycling, a 500-μm-long double-T channel junction with a tapered structure for post-PCR cleanup and inline injection,[46] and a 14-cm-long channel for CE separation. These two systems share an anode, a cathode, and a waste well to reduce the number of reservoirs on the chip.

The microdevice is constructed using a four-layer wafer stack consisting of (from top to bottom) glass manifolds, PDMS membranes, a glass fluidic wafer, and a glass RTD wafer. The microfabrication process has previously been described in detail.[44, 51] Prior to use, the microchannels are coated with 0.25% polyDuramide (pDuramide) dynamic coating polymer to minimize DNA absorption to the channel walls and electroosmotic flow during electrophoresis. The coating procedure consists of 1 M HCl incubation for 15 min, DI water flush, and pDuramide incubation for one hour. After treatment, the chips are flushed with water again, and then dried with vacuum.

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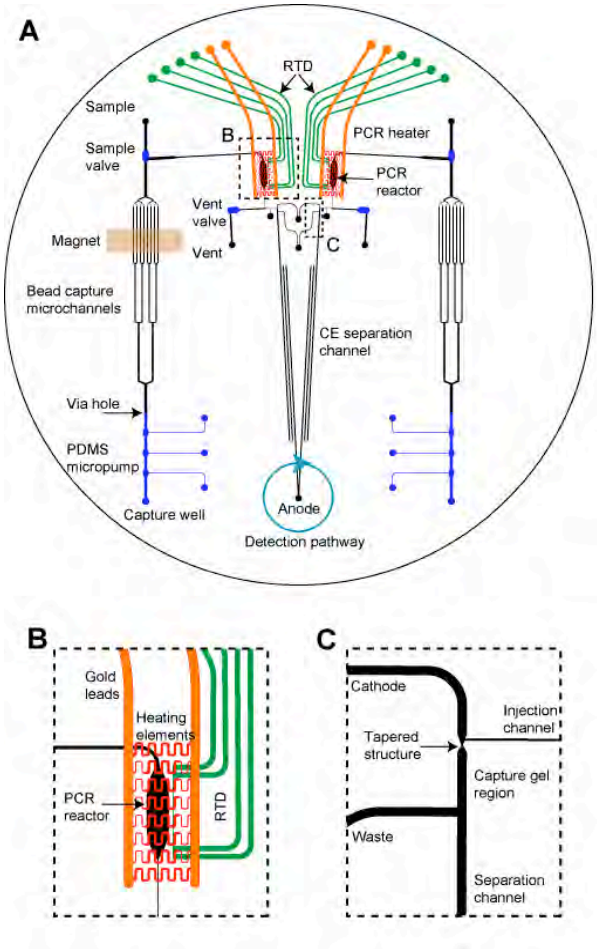

Figure 19. The design of the fully integrated microdevice for forensic STR analysis. (A) Mask design for the microchip capable of performing DNA template capture, PCR, capture inline injection and CE separation. The microchannels are indicated in black, the microfabricated RTD and electrodes are in green, the heater is shown in red, the gold leads of the heater are drawn in gold, and the PDMS micropumps and microvalves are in blue. (B) Expanded view of the PCR chamber and the microfabricated heater and RTD. (C) Expanded view of the capture inline injector with a tapered structure.

STR typing and DNA capture probes. A 9-plex autosomal STR typing system was developed previously based on the primer sequences and fluorescence dye labeling scheme used in PowerPlex® 16 System (Promega, Madison, WI).[44, 46] To enable the post-PCR cleanup and inline injection, the unlabeled primers were replaced with biotin-labeled primers (IDT, Coralville, IA). The STR loci included in the 9-plex system are amelogenin for sex typing and 8 CODIS core STR loci (D3S1358, TH01, D21S11, D5S818, D13S317, D7S820, vWA and D8S1179). The biotin-labeled primers in the 9-plex STR typing systems were also employed as capture probes for DNA template capture prior to PCR. The concentrations of the capture probes in a 10× capture probe mixture are listed as follows: Amelogenin: 27 nM, D3S1358: 17 nM, TH01: 22 nM, D21S11: 43 nM, D5S818: 11 nM, D13S317: 13 nM, D7S820: 47 nM, vWA: 13 nM and D8S1179: 11 nM. The capture probe mixture was incubated with genomic DNA for liquid-phase DNA hybridization, and then captured by streptavidin-coated magnetic beads via streptavidin-biotin binding on the chip.

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DNA sample preparation. Standard genomic DNA 9947A, 9948 and K562 were purchased from Promega and diluted in deionized water (DI water). To determine the heating time for fragmenting genomic DNA, K562 DNA (0.4 μg/μL) with high molecular weight was subjected to 95°C heat incubation for different times (from 10 to 40 min in 5-min intervals) in a PTC-200 thermocycler (MJ Research, Waltham, MA). DNA samples are then run on a 1.2 % agarose gel with 100-4 kb and 1-4 kb sizing ladders (Lonza, Allendale, NJ). These results demonstrate that 20-min heating is sufficient to fragment the genomic DNA into a size range of 1-4 kb. To prepare DNA samples for on-chip template capture, 20 μL DNA samples were first heated at 95 ^{o}C for 15 min in a PTC-200 thermocycler. After mixing the fragmented DNA with 25 uL of 20× sodium saline citrate (SSC) buffer and 5 uL of 10× capture probe mixture, the solution was further heated to 95 ^{o}C for 5 min, followed by incubation at 50 ^{o}C for 20 min to allow DNA hybridization between the biotin-labeled capture probes and the target DNA fragments.

Microchip operation. Following the photopolymerization of the capture gel plugs in the chip using the method developed previously, a separation matrix (5% LPA with 8 M Urea in 1× TTE) is loaded from the anode to the waste and from the cathode to the coinjection reservoir to form a matrix-capture-matrix gel sandwich structure in the capture inline injection regions. The tapered structure in the capture region ensures that the capture gel plug will be retained. After gel loading, a 15 min incubation with 1% w/v BSA solution followed by acetonitrile rinse is conducted. SSC (10×) is finally loaded into the bead capture channels while keeping the vent microvalve closed to prevent SSC buffer from getting into the PCR reactors.

The DNA template capture process begins by introducing 5 μL of prepared Dynabeads (Invitrogen, Carlsbad, CA) solution (~1.3×10^{6} beads) into the capture well. The magnetic beads are driven into the capture structure using the on-chip micropump and immobilized in the microchannels using a nickelplated neodymium magnet (All Electronics, Van Nuys, CA). A multi-step loading procedure is employed to establish equal bead distributions across all parallel capture channels.[50] DNA solution (20 μL) containing fragmented genomic DNA and capture probes is then pumped through the capture structure using a 9-step pumping protocol with three "flutter" steps (200 ms/step

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for pumping and 100 ms/step for fluttering).[50, 51] Following sample loading, 10 μL PCR cocktail containing all the necessary components for PCR except DNA template is used to wash the bead bed. The bead bed is then pumped into the PCR chamber by closing the sample valve, opening the waste valve, and placing the magnet above the reactor.

Once the magnetic beads are loaded into the PCR reactor, ten more microliters PCR cocktail is loaded into the capture well and pumped through the beads. After that, the PCR thermal cycling begins with all valves held closed at 20 kPa. The modified thermal cycling protocol starts with an initial activation of the Taq polymerase at 95 ^{o}C for 4 min. For the next 32 PCR cycles, the temperature is held at 94 ^{o}C for 10 s denaturing, then ramped to 58 ^{o}C for 20 s annealing, and then to 70 ^{o}C for 30 s extension. Finally, a post extension step is performed at 70 ^{o}C for 5 min. Total PCR time is 40 min.

To perform the purification, the sample valve is held open and the biotin-labeled PCR products are electrophoretically injected using an electrical field of 25 V/cm from the PCR chamber to the waste well through the capture gel plug. The products are bound via the biotin-streptavidin interaction to form a tightly concentrated plug in the capture gel. Unbound materials are washed away in the washing step. The fluorescently labeled DNA strands retained in the capture gel are then thermally released into the separation channel by heating the whole chip to 67 ^{o}C and applying an electrical field of 250 V/cm towards the anode. After each run, all the gels and solutions in the chip are removed out with water and the channels and chambers are cleaned using piranha (7:3 H_2SO4: H_2O_2) to prevent run-to-run carryover contamination.

Genomic DNA digestion. To enable the sequence-specific DNA capture, genomic DNA with high molecular weight must be fragmented into an appropriate size range which can be captured by magnetic beads while still providing intact templates for subsequent PCR amplification. DNA digestion by heating was chosen in this study because it can be rapidly performed in a conventional thermal cycler. Moreover, since the DNA extraction process in forensic sample analysis already includes a heating step for cell lysis, the heat digestion can be easily incorporated into the extraction procedure without any additional operation. As shown in Figure 20, heating DNA samples at 95 ^{o}C for 20 min is sufficient to fragment genomic DNA into a size range of 1-4 kb.

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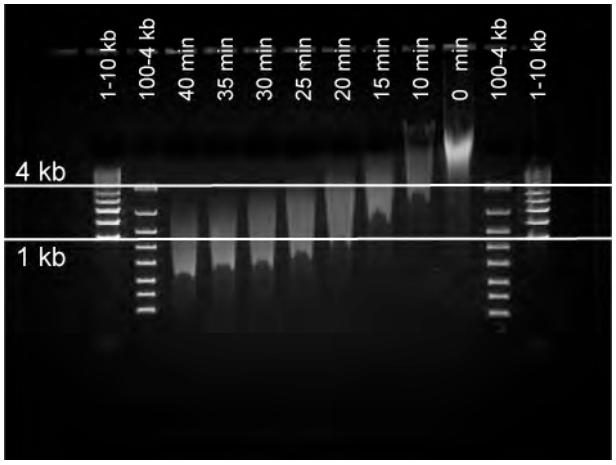

Figure 20. Photograph of gel electrophoresis separation of genomic DNA thermally fragmented for different lengths of time. K562 standard genomic DNA with high molecular weight was subjected to 95°C heat incubation for different times and run on a 1.2 % agarose gel. 20-min heating is sufficient to fragment the genomic DNA into a size range of 1-4 kb.

Sequence-specific DNA capture. The evaluation of the efficiency of the DNA template capture was performed on the glass microchip which only contains bead capture microchannels coupled with a PDMS micropump. To quantify DNA capture by the Dynabeads, real-time PCR is performed on ABI 7300 instrument (Applied Biosystems, Foster City, CA) using a SYBR® Green PCR Master Mix (Applied Biosystems). Since the capture probes may have different capture efficiency for different STR loci, the concentrations of these probes were iteratively adjusted to ensure balanced template capture for all 9 STR loci. As shown in Figure 21, the capture efficiencies for these 9 STR loci are in a range of 3.7 - 7.0 % with an average of 5.4 ± 1.3 %. Although this efficiency is lower than those of many stand-alone DNA extraction microdevices developed previously,[52-54] the 100% transfer of the captured DNA to the PCR reactor compensates for this drawback and makes the sequence-specific DNA capture suitable for high-sensitivity STR typing.

Another advantage provided by sequence-specific DNA extraction is the improved PCR efficiency due to the complete removal of background DNA sequences. Figure 22 presents 9-plex STR profiles amplified from 1 ng of whole genomic DNA (9948) and from ~ 1 ng DNA fragments purified from genomic DNA by sequence-specific DNA capture. The PCR amplifications were performed in a conventional thermal cycler with a PCR protocol which includes denaturation at 94 ^{o}C for 10 s, annealing at 58 ^{o}C for 20 s, and extension at 70 ^{o}C for 30 s. Compared to the PCR protocol recommended by the manufacturer, over 1.5 hr is saved.[55] Using this shortened

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cycling, full STR profiles still can be obtained from the purified DNA. In contrast, the profiles from the whole genomic DNA experienced dropout of the TH01 9.3 allele. This comparison demonstrates the effectiveness of sequence-specific DNA purification for improving STR amplification.

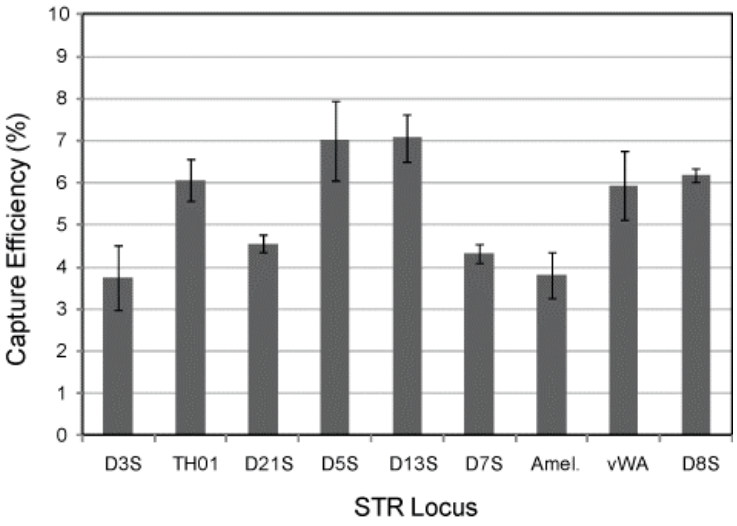

Figure 21. The capture efficiency of the sequence-specific DNA template purification process. By optimizing the capture probe concentrations, similar capture efficiencies with an average of 5.4% were obtained for all 9 STR loci.

This improved PCR efficiency is extremely useful in the quest of expediting forensic STR typing. In current STR amplifications, high annealing temperatures, long holding time and slow temperature ramping rate are usually employed in order to ensure balanced and highly stringent amplifications of all STR loci and to avoid non-specific amplicons. For example, the Powerplex 16 STR kit recommends 60 ^{o}C for annealing and a temperature ramping rate of 0.5 ^{o}C/s from denaturation to annealing, resulting in a PCR protocol longer than 3 hours.[55] In our previous study of on-chip STR amplifications, we also found that the slow ramping rates are critical for generating reproducible and balanced STR profiles.[45] Faster temperature transitions resulted in allele dropout and imbalanced profiles. The on-chip PCR time was thus only shortened to 2 hours. Sequence-specific DNA template capture is an effective way to overcome this hurdle for rapid STR amplification. The removal of unnecessary DNA sequences eliminates the associated potential non-specific amplification, adding stringency prior to the PCR step. Under these conditions it is possible to use a lower annealing temperature allowing more efficient primer binding together with a faster ramp rate that accelerates the PCR process.

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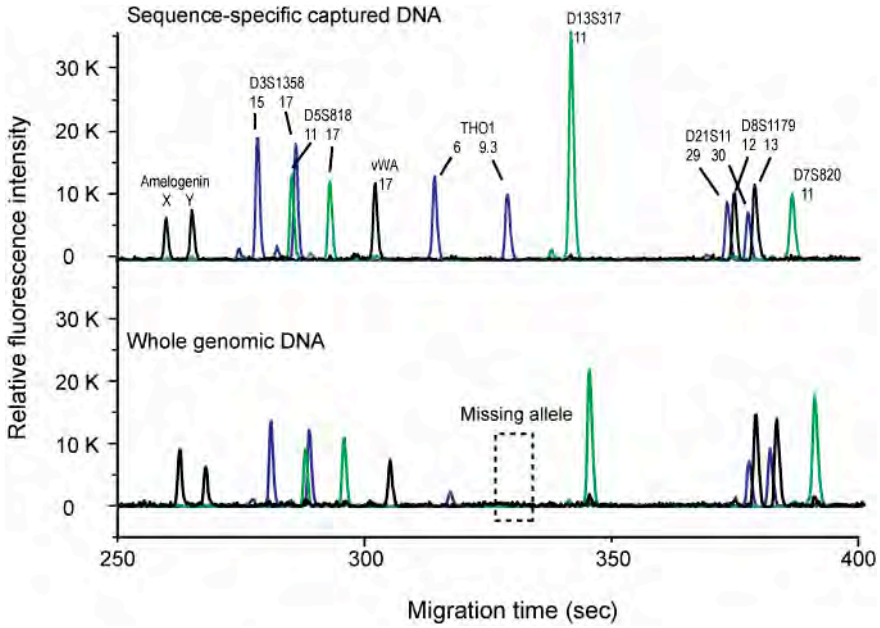

Figure 22. Comparison of the 9-plex STR profiles obtained from whole genomic DNA and purified DNA. A total of 1 ng whole genomic DNA and ~1 ng DNA fragments purified from genomic DNA by sequence-specific DNA capture were amplified in a conventional PTC-200 thermocycler with a PCR protocol consisting of denaturation at 94 ^{o}C for 10 s, annealing at 58 ^{o}C for 20 s, and extension at 70 ^{o}C for 30 s. With these shortened heating times for PCR cycling, the DNA sample purified by sequence-specific DNA capture still provides full STR profiles. However, in the profiles from the whole genomic DNA, the TH01 9.3 allele drops out.

Limit of detection. DNA template capture, 9-plex autosomal STR amplification, post-PCR cleanup, capture inline injection, and CE separation on the fully integrated microsystem were tested using standard female 9947A genomic DNA. The limit-of-detection of the microsystem was also explored using serially diluted 9947A DNA. The obtained STR profiles were assessed using the allele calling protocol developed previously.[26] As shown in Figure 23, full DNA profiles can be obtained with as few as 2.5 ng input DNA. In our previous study, the limit of detection of the PCR-CE microdevice was determined as 100 copies of DNA templates (0.333 ng) in the 160-nL PCR reactor. However, a 10-μL PCR solution containing ~21 ng DNA is needed to load 0.333 ng DNA into such a small-volume PCR reactor. The integration of the bead capture structure successfully concentrates the DNA template into the PCR reactor. Considering the average 5.4% capture efficiency of the sequence-specific purification, about 45 copies of DNA were purified from the 2.5 ng input and loaded into the PCR

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chamber for the following amplification, post-PCR cleanup, inline injection and CE detection. The detection limit of 45 copies for PCR amplification is already approaching the low-copy-number range (≤ 100 pg or 33 copies of each locus).

The total analysis time of this assay on the microsystem is about 2 hours and 35 min, which includes 40 min of DNA digestion and hybridization, 40 min of DNA template capture and washing, 45 min of PCR amplification, and 30 min of post-PCR cleanup, capture inline injection and CE. Compared to conventional forensic STR typing (7-8 hours), at least 3-4 hours can be saved by using this system. Therefore, this microsystem can be utilized as an automated instrument for rapid forensic STR analysis in forensic laboratories.

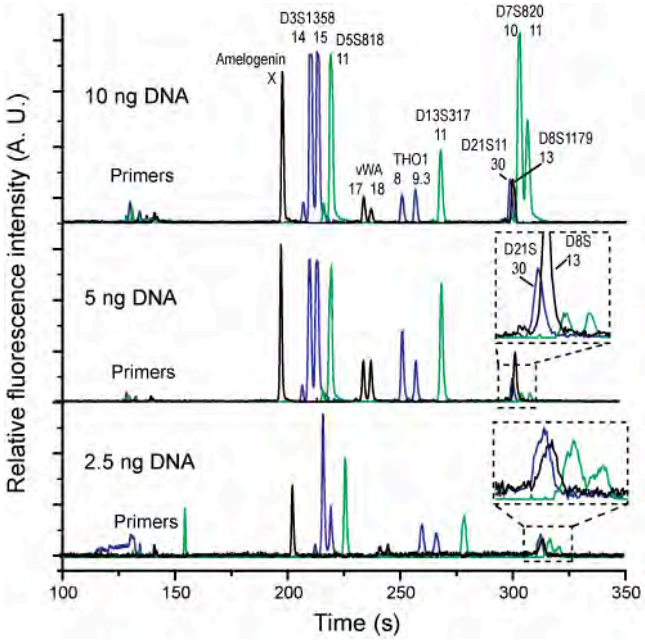

Figure 23. The Limit of detection test of the microsystem using serially diluted female 9947A DNA. With only 2.5 ng DNA input, full DNA profiles can be obtained successfully.

In conclusion, a fully integrated microdevice capable of performing DNA purification, PCR, post-PCR cleanup, and CE separation using a compact detection and control instrument and chip was successfully developed for rapid forensic STR analysis. Efficient sample transfer between each function units was achieved by the integration of sequence-specific DNA template capture using magnetic beads and post-PCR capture inline injection. The operation of this microdevice was optimized using standard DNA samples, validating the feasibility of rapid STR analysis on an integrated microfluidic system. This study is a significant step towards a fully integrated and portable forensic

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analysis system for rapid real-time human identification at crime scenes or other point-of-analysis situations. In the future, the adoption of a 16-plex STR typing system is desirable. Additionally, the operation of this integrated system should be further simplified so that forensic scientists with minimal training can successfully apply this technology in routine forensic analysis.

5. Development of 2nd Generation μCAE Scanner

All of our work summarized above was supported by the development of the 2nd generation bench-top rotary μCAE scanners with reduced size and operational complexity for integrated STR analysis. We successfully designed and constructed a compact scanner system that contains the classical multi-color rotary scanning confocal detection system together with sufficient pneumatic and electronic components to provide a general compact portable platform for the future development of fully integrated microfluidic systems The Multi-channel Capillary Array Electrophoresis Portable Analyzer (McCAEPS) is shown in Figure 24A. The instrument was developed to perform μCAE analysis alone,[26, 56] cleanup and separation of off-chip amplified samples,[46] on-chip PCR and electrophoretic analysis,[44, 45] as well as fully integrated sample-in-answer-out assays in the future. The separation of off-chip amplified STR samples using a 96-lane μCAE microplate and the analysis of 100 copies of standard 9948 DNA using an integrated PCR-CE device were conducted to verify the performance of the instrument. Results of this work and the technical details are being prepared for submitting to *Review of Scientific Instruments.* A summary of the technical details of this work is provided in the following.

Instrument structure. The instrument contains a confocal optical system with a rotating objective for detecting four different fluorescence signals, pneumatics for control of on-chip microvalves and micropumps,[41] four PCR temperature control systems,[57] four high voltage power supplies and one grounded electrode for electrophoresis. The instrument has dimensions 12 × 12 × 8 in. with a total weight of 28 lbs, and it can be used as either a bench-top or portable instrument. An exploded view of the instrument is shown in Figure 24B. The instrument has two sections divided by a main support plate that is supported by two side support plates. A cover plate is attached to the top of the

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two side panels and to the optics support plate with a small support rod to prevent bowing of the top plate.

Optical design. The optical system (scaled illustration) is shown in Figure 24C. The instrument uses a solid-state 488-nm laser (Saphire-488, Coherent, Santa Clara, CA) delivering 100 mW of power to excite fluorescence from labeled DNA fragments. The laser beam is reflected with two dielectric mirrors up through a dichroic mirror (Chroma, Rockingham, VT, Z488bpxr). It then passes through the hollow shaft stepper motor and enters the bottom plate of a three-part balanced rhomb assembly that sandwiches the rhomb between top and bottom plates and holds the objective. The beam is displaced 7 mm by the rhomb, and is focused into the center of the microplate channel. The low mass of the rhomb objective assembly (48g) allowed us to use a small hollow shaft rotary motor (Empire Magnetics, Inc. U17-7). The clear aperture of the objective was 2.8 mm, which was small enough to pass through the hollow shaft of the stepper motor. Fluorescence collected by the objective returns through the stepper motor shaft and is reflected by the dichroic mirror into a confocal assembly. The light is focused on a 200 μm pinhole with a 20mm fl achromat lens, collimated into a 0.7mm diameter beam with a 5mm fl achromat lens, passed through a longpass filter with OD of 8 at 488nm (Chroma, HHQ505lp) and enters the 4 color PMT (Hamamatsu H9797 fitted with special sequential dichroic beam splitter optics Chroma, 537dclp, 570dc, 595dclp, Z488bpxr). The detection system collects 5000 data points per revolution of the objective at 5 Hz giving an integration time of 40 μs/point over a distance of 8.796 μm. The instrument was designed to take data from 96 channels in a 150 mm diameter microplate or 384 lanes in a 203mm diameter microplate. We have constructed two miniature rotary scanners that differ principally in the size of the heated microplate holder and the placement of the optics.

Electronic components. A view of the instrument from the bottom with the bottom plate folded down is shown in Figure 24D. The electronics compartment holds 4 high voltage power supplies (one 3KV, three 1KV) and one spare grounded electrodes, 4 sets of PCR heater controls, 5 signal sensing and conditioning boards for PMT output and trigger channels, stepper motor controller EZHR17 (All Motion, San Jose, CA), and laser controller board. The PMT output is conditioned with 12 kHz low pass filters and the

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output from the RTD PCR sensors is conditioned with 5 Hz low pass filters. The instrument is controlled with a NI 6259 OEM multifunction DAQ board (National Instruments, Austin, TX) that has 32 analog inputs (16bit), 4 analog outputs and 48 digital I/O lines and a laptop computer. A separate power supply operating on 120V is used to supply the instrument with 12 V and ±15V DC and 120V AC. The maximum power consumption of the instrument is 380W.

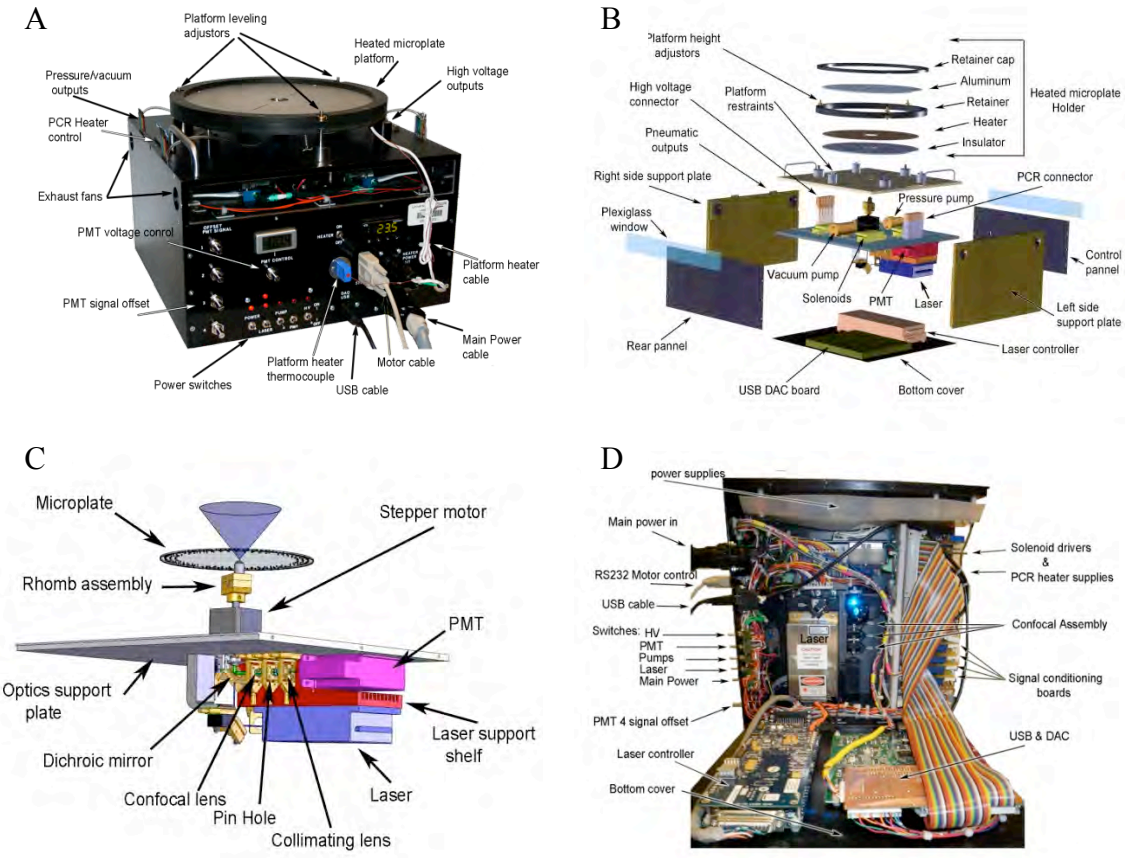

Figure 24. (A) Photograph of the McCAEPS. (B) Exploded view of the instrument. (C) Schematic of the 4-color confocal detection system with a rotary objective. (D) Bottom view of the open instrument.

Pneumatic system. The top of the main support plate holds two vane rotary pumps (Thomas, G 12/02-8-LC 15V, Vacuum; G12/02-8 Pressure) and 4 manifolds (LHLX0500200BB) each holding 8 latching solenoids LFMX0510533B, (The Lee Company, Essex CT). One of the outputs in each bank was modified to provide vacuum to hold the microplate flat against the aluminum support plate and inject ambient air

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instead of pressure to release it. This configuration enables us to apply vacuum hold/release to four sections of the microplate independently and hold 100mm diameter microplates in one quadrant of the microplate holder. The pneumatic lines from each solenoid were connected with tubing to inlets on the inside of the support panels that lead to exit ports on the outside top of each support plate. These pneumatic lines are attached via 1/8" tubing to any desired configuration of input ports on manifolds that rest on the microplate to control on-chip microvalves and pumps.

Microchip platform and manifolds. The heated microplate platform was designed to sandwich a 3mm thick Aluminum plate, diameter 226.3 mm, that is heated on the bottom with a 250 W, 120V Kapton heater (Durex Industries, Cary, IL, KP1098-01 with foil and PSA backing). A thermocouple is imbedded into bottom of the aluminum support plate and has its sensing element approximately .5 mm from the top surface. A 1/8" neoprene insulation layer covers the underside of the heater. The sandwich design minimizes vertical displacement or bowing that could change the position of the focus in the channel when heated from ambient to 70 ^{o}C. The heated microplate holder is held firmly with extension springs to three kinematic support posts (flat, cone & V groove). Three other adjustable stops in mirrored positions keep the holder from being lowered to where the objective would collide with the microplate.

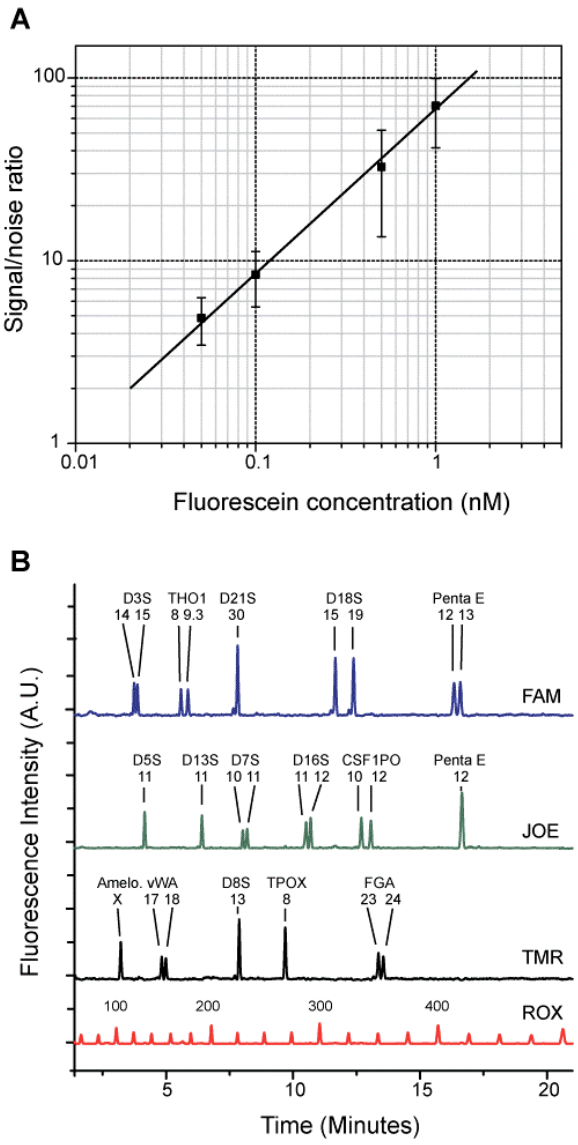

Figure 25. (A) The signal to noise as a function of fluorescein concentration. The limit of detection of

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the system is determined as ~20 pM with a S/N of 2. (B) Representative PowerPlex 16 profiles amplified from 1 ng of 9947A female standard DNA obtained using the 96-lane μCAE microchip on the instrument.

Limit of detection and capillary electrophoresis. A sensitivity study using serially diluted fluorescein dye was carried out to determine the detection limit of the scanner system. The fluorescein dye with a concentration range of 0.05-1 nM (pH 8) was separated on a 96-lane μCAE microdevice fabricated using the published procedures.[26] Figure 25A plots the S/N as a function of the concentrations of fluorescein dye, demonstrating the detection limit of this system is ~20 pM fluorescein with a S/N of 2. The separations of STR samples amplified off-chip using the 96-lane μCAE microdevice were also conducted to verify the operation of capillary array electrophoresis using the instrument. As shown in Figure 25B, the fully resolved DNA profiles demonstrate that the McNAPA can provide similar performance for on-chip μCAE analysis as the proof-of-concept setup built previously by our group.

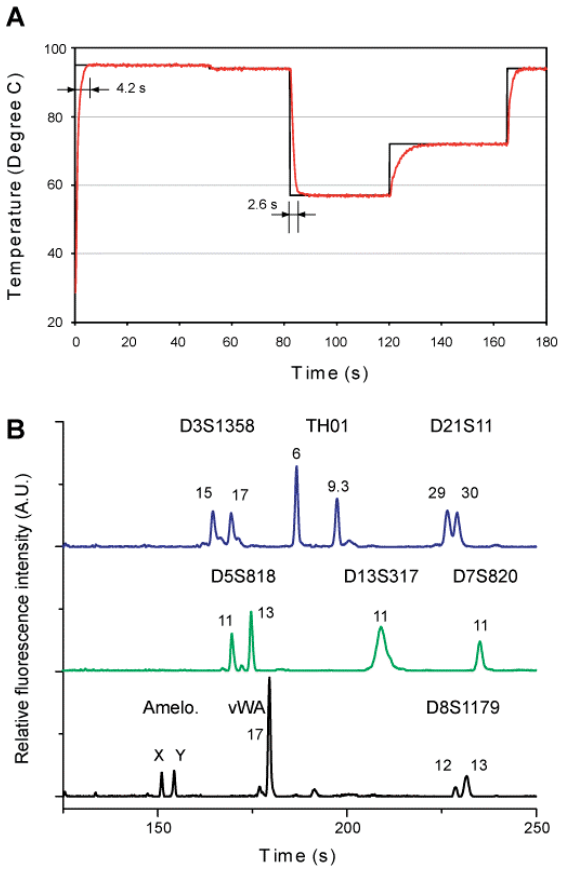

Figure 26. (A) Thermal cycling amplification profile. Red line shows the measured temperature from the RTD and black line is the set temperature. Temperature ramp rates were 16 °C /s for heating and 13.8 °C /s for cooling. (B) DNA profiles amplified from 100 copies of 9948 male standard DNA using the integrated PCR-CE microsystem.

Integrated PCR and electrophoresis. We also conducted an integrated PCR and capillary electrophoresis test using the PCR-CE microdevice developed previously in our group to fully characterize the electronic and pneumatic systems of the McCAEPS. The 4-inch-diameter PCR-CE microdevice consists of a 160-nL PCR chamber, an microfabricated heater, a four-point resistance

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temperature detector (RTD), two poly(dimethysiloxane) (PDMS) microvalves, and a 7-cm-long CE separation channel.[44, 45] In this study, a10-μL PCR solution containing ~21 ng DNA is prepared to load 100 copies of DNA into the PCR chamber for thermal cycling. Figure 26A shows a temperature cycling profile recorded from this microchip. The temperature ramp rates can reach 16 °C/s for heating and 13.8 °C/s for cooling. As shown in Figure 26B, the 9948 DNA profiles obtained by this microsystem prove that the McNAPA can be used as a general platform for integrated microfluidic devices which require precise temperature control, fluid manipulation, and high-sensitivity fluorescence detection.

In conclusion, we have successfully developed a compact, self-contained instrument for control and detection of high-throughput integrated microfluidic microdevices. Several unique features make this instrument a versatile platform for genetic analysis: (i) the 4-color rotary confocal detection system as well as the 226.3-mm-diameter heated platform can support microchips with throughputs from single channel on 4" wafers up to 384 channels on 8" wafers; (ii) four independent temperature control systems coupled with microfabricated heaters and RTDs provide rapid, localized heating which is required by STR amplification; (iii) a total of 28 solenoid valves providing pressure/vacuum connections can realize complicated microfluidic manipulation using PDMS microvalves and micropumps. In our laboratory, this instrument will enable the development of more challenging studies in forensic STR analysis, such as high-throughput STR typing from single cells. The presentation of the design and fabrication details will also enable others to exploit the unique elements of this instrument.

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DISSEMINATION

Publications:

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Commercial dissemination:

In addition to these traditional dissemination mechanisms, Mathies and U.C. Berkeley have worked with a local company originally named Microchip Biotechnologies, Inc. (MBI) and now named IntegenX in Dublin, CA. IntegenX has licensed many of the technologies described in this report and with funding from In-Q-Tel and other private sources has developed a fully integrated system for forensic identification that is being evaluated by various defense intelligence agencies as well as the FBI and NIST. The IntegenX website is http://integenx.com for more information on the success of this project.

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