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A fully integrated and automated microsystem for rapid pharmacogenetic typing of multiple warfarin-related single-nucleotide polymorphisms

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Manuscript Information: Pages: 27; Figures: 8.

Abstract words: 240.

Total words: 7011.

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Abstract:

A fully integrated and automated microsystem consisting of low-cost, disposable plastic chips for DNA extraction and PCR amplification combined with a reusable glass capillary array electrophoresis chip in a modular-based format was successfully developed for warfarin pharmacogenetic testing. DNA extraction was performed by adopting a filter paper-based method, followed by an "*in-situ*" PCR that was carried out directly in the same reaction chamber of the chip without elution. PCR products were then co-injected with sizing standards into separation channels for detection using a novel injection electrode. The entire process was automatically conducted on a custom-made compact control and detection instrument. The limit of detection of the microsystem for the singleplex amplification of amelogenin was determined to be 0.625 ng K562 standard DNA and 0.3 μL of human whole blood. A two-color multiplex allele-specific PCR assay for detecting the warfarin-related single-nucleotide polymorphism (SNP) 6853 (-1639G>A) and 6484 (1173C>T) in the *VKORC1* gene and the *3 SNP (1075A>C) in the *CYP2C9* gene was developed and used for validation studies. The fully automated genetic analysis was completed in two hours with a minimum requirement of 0.5-μL input blood. Samples from patients with different genotypes were all accurately analyzed. In addition, both dried bloodstains and oral swabs were successfully processed by the microsystem with a simple modification to the DNA extraction and amplification chip. The successful development and operation of this microsystem establishes the feasibility of rapid warfarin pharmacogenetic testing in routine clinical practice.

Key words: Pharmacogenetic Testing, Microfluidics, micro-Total Analysis System, Single-Nucleotide Polymorphisms

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INTRODUCTION

The traditional "one-size-fits-all" approach in drug prescriptions may well be reaching its end days as more is learned about diseases and genes, and more drugs are developed using a biomarker-based method.[1-3] The adoption of pharmacogenetic testing in clinical practice will enable the use of medications based on patients' genetic information (i.e. personalized medicine), resulting in improved safety and efficiency.[4, 5] One of the well-known examples is warfarin, which is the most widely prescribed anticoagulant drug in the US.[6, 7] Due to a narrow therapeutic window and large inter-patient variability in drug sensitivity, thrombosis and bleeding caused by inappropriate warfarin dosages have been identified as the second leading cause of drug-related emergency room visits.[8, 9] Although many factors, such as age and life style, influence the warfarin response,[9] single-nucleotide polymorphisms (SNPs) in the *CYP2C9* (cytochrome P450, family 2, subfamily C, polypeptide 9) and *VKORC1* (vitamin K epoxide reductase, complex 1) genes account for approximately 40% of the inter-patient variability in the warfarin dosage.[10] Several independent studies have found that the consideration of patients' *CYP2C9* and *VKORC1* genotypes when prescribing the drug significantly reduced hospitalizations up to 43% compared to the non-genotyped groups.[6, 11, 12] Despite compelling findings and clinical potentials, incorporation of pharmacogenetic testing into routine clinical practice to guide treatments is still restricted due mainly to the accessibility and the time-consuming nature of the current existing diagnostic methods for probing these genetic variations.[13]

The current pharmacogenetic testing for detecting SNPs or mutations is mainly constrained by sample preparations, which usually include nucleic acid extraction from clinical samples and amplifications of target sequences by polymerase chain reaction (PCR).[14, 15] Even the detection approaches, such as microarray, capillary electrophoresis (CE), real-time PCR, and DNA sequencing, have been significantly improved in

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sensitivity and automation, the entire analytical process starting from a raw sample to a result is still time-consuming, labor-intensive, and high-priced.[13, 16] Microfluidic technology holds great promise to address these problems by integrating the sample preparation steps with the detection together to form a micro total analysis system (μTAS).[14, 17, 18] In past decades, a variety of fully integrated microsystems based on different detection methods have been developed. For instance, the Cepheid GeneXpert system (Sunnyvale, CA) that integrated sample preparations with real-time PCR was developed for the diagnosis of tuberculosis. However, in the case of SNP and mutation detections, the capillary electrophoresis-based system is believed to be one of the best choice due to its capability of the multi-locus detection, the degree of total integration, and the technology readiness level.

The first sample-in-answer-out microsystem based on the electrophoretic detection was reported by Landers' group, in which a solid-phase extraction (SPE) column for DNA extraction was integrated with PCR and CE on a single device for pathogen detection.[19] Meanwhile, Mathies' group has successfully demonstrated PCR-CE glass devices for pathogen detection and forensic DNA typing.[20-22] Later, they introduced a template purification and a post-PCR purification structures into the microsystem for an automated DNA typing from buccal swabs.[23, 24] In collaboration with the Forensic Science Service of UK, Zenhausern's group of the University of Arizona described a prototype instrument that consisted of a plastic extraction and amplification cartridge coupled with a glass CE microchip for rapid forensic short tandem repeat (STR) analysis from pre-prepared lysates of buccal swab samples.[25] More recently, IntegenX (Pleasanton, CA) and GE Healthcare (Pittsburgh, PA) released their fully automated RapidHIT™ and DNAScan™ systems, respectively.[26-28] Both instruments can complete a STR typing in less than 90 minutes. In addition, MicroLab Diagnostics collaborated with Landers' group reported a new prototype that is capable of performing automated DNA typing

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from buccal swabs or FTA® papers.[29, 30] While the emergence of so many new systems and products clearly indicates the era of the "sample-in-answer-out" genetic analysis is approaching, the assays performed on these systems are still focused on forensic human identification by far, and the cost per assay is still quite expensive (> $250 per sample). With further improvement in the cost and the versatility, we believe that this extraordinary technology will play an important role in the field of pharmacogenetics.

Here we presented a fully integrated and automated microsystem consisting of disposable plastic chips for DNA extraction and amplification coupled with a 4" reusable glass array-CE chip in a modular-based integration format, operated on a compact control and detection instrument for rapid pharmacogenetic testing. A filter paper-based DNA extraction followed by an "*in-situ*" PCR amplification was conducted in the DNA extraction and amplification chip.[31, 32] After that, amplicons were driven to an injection chamber, where a novel needle-shape injection electrode was employed to mix and deliver the amplicons with sizing standards to the CE chip for separation and detection. To demonstrate the potential application of the system, we performed the detections of three single-nucleotide polymorphisms in *CYP2C9* and *VKORC1* from whole blood, bloodstains, and buccal swabs. This work will promote the future use of rapid genetic analysis in clinical practice for guiding the prescription of drugs according to patients' genetic information.

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Experimental Section

Microdevice design and microfabrication

The fully integrated microchip assembly is comprised of three separated microdevices: two identical plastic DNA extraction and amplification chips (DEA chip) and one glass capillary array electrophoresis chip (CAE chip), which are combined together in a modular-based integration format. As shown in Figure 1A, the structure of the DEA chip is similar to that of the sample preparation chip developed previously in our group.[32] Briefly, the DEA chip consists of two PMMA layers with sandwiched PDMS membrane discs (BISCO® HT-6240, Rogers, Woodstock, CT) for valve actuation, filter paper discs (Fusion 5, GE Healthcare, Pittsburgh, PA) for DNA extraction, and sealing tapes for chamber isolation. Two DNA extraction and amplification units, each of which contains an extraction inlet, a shared PCR inlet, a waste outlet, a loading chamber, a reaction chamber, an injection chamber, and three mechanically actuated diaphragm microvalves, are symmetrically arranged on the DEA chip. The loading chamber is a 3-mm-diameter well for accommodating a variety of raw sample materials. According to different sample types, a piece of filter net with a mesh size of 80 μm is embedded into the chamber for carrying whole blood or a simply empty well is designed for housing dried bloodstains and buccal swabs. The 15-μL reaction chamber with a 3-mm-diameter piece of Fusion 5 filter paper disc is designed for DNA extraction and *"in-situ"* PCR amplification. Three microvalves (Valve 1, 2, and 3) are employed to switch flows on/off to the reaction chamber during the extraction and to seal the chamber completely while thermal cycling. The injection chamber is constructed by aligning two 3-mm-diameter via holes together, which are located in the upper and the lower PMMA layers, respectively. A piece of sealing tape (Microseal® B adhesive tape, Bio-Rad, Hercules, CA) is sandwiched between two via holes to divide the injection chamber into two compartments: the upper compartment for receiving PCR products transferred from the reaction chamber, the lower one for storing pre-mixed sizing standards and formamide which are preloaded

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into the DEA chip, and later co-injected with PCR products to the CAE chip. Two pieces of BarSeal™ tape (Thermo Fisher, Waltham, MA) are applied to enclose the injection chamber from both sides of the chip.

The design of the CAE chip, as shown in Figure 1B, is similar to those that have been published elsewhere.[23, 33] On a 4" glass wafer, four 16-cm-long separation channels with a cross-section of 200 ×40 μm are grouped into two doublets. Each doublet contains two double-T injectors with an offset of 200 μm, two sample wells, a cathode, a waste, and an anode wells. Due to the space limit of the chip, tapered turns are employed to extend the separation channels up to 16 cm with minimized diffusion disturbance during electrophoresis. Fabrication of the glass chip follows procedures previously described.[23, 33] Two 3-mm-thick PDMS blocks with punched buffer reservoirs are permanently bonded to the glass CAE chip using a plasma treating method. To assemble the fully integrated microchip system, as shown in Figure 1C, two DEA chips are reversibly attached to the PDMS blocks on the CAE chip by aligning four injection chambers to the corresponding sample wells. A non-leaky contact between the PDMS and the plastic DEA chips can be easily obtained with a simple pre-cleaning of the PDMS surface.

As shown in Figure 2, a novel injection electrode coupled with the injection chamber of the DAE chip was developed to enable the sample transfer from the DAE chip to the capillary electrophoresis, as well as the mixing of PCR products with the injection reagents. The steel electrode with a cross groove is coated with a layer of platinum to prevent electrochemical reactions. The injection is realized by actuating the electrode to punch through three layers of tapes in the injection chamber completely. Due to the capillary action generated by the groove, PCR products as well as the sizing standards and formamide are aspirated into the electrode, where all

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the solutions are well mixed together. After that, an electrical field is applied between the injection electrode and the waste electrode to deliver the prepared samples to the injection channel for electrophoresis.

Instrumentation for microchip control and detection

A photograph of the instrument used to perform the fully automated pharmacogenetic testing is shown in Figure 3A. The system with dimensions 48×35×35 cm contains a 488-nm diode laser (100 mW, Sapphire 488, Coherent, Santa Clara, CA), a confocal optical system with a linear-motion objective for detecting four different fluorescence signals, four high voltage power supplies for electrophoresis, a fluidic control system, a microchip fixture, and all the electronics for system operation. A custom-made LabVIEW program (National Instruments, Austin, TX) running on a laptop is used to control the system through a NI 6259 OEM multifunction DAQ board (National Instruments).

The design of the optical system is identical to that developed previously in our group.[32] Briefly, the laser beam is reflected by two mirrors, and then transmitted through a dichroic mirror into a linear-motion assembly that is mounted on a motorized scanning stage. The laser beam is further reflected by a prism in the assembly and focused to the microchip by an objective. Excited fluorescence is collected by the objective, and then reflected by the prism and the dichroic mirror into a four-color confocal system, where the light is directed into a multi-channel PMT (photomultiplier tube) with four detection wavelength ranges centered at 517, 549, 576, and 605 nm. The converted electrical signals are collected at a rate of 20 kHz by the DAQ board and processed using a 10-Hz low-pass filter in the LabVIEW program. The fluidic control system has also been described in details previously.[32] In the instrument, a tube rack that provides sodium hydroxide (NaOH) for cell lysis,

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hydrochloric acid (HCl) for neutralization, 1× TE (Tris EDTA) and DI water for rinsing, is installed in the front side of the instruments (Figure 3B). A syringe pump (PSD/4, Hamilton, Bonaduz, Switzerland) and a modular valve positioner (MVP) (Hamilton) are employed to realize the fluid pumping and switching functions, respectively. The fluids are routed to the microchip fixture located on the top of the system via PTFE tubing.

The microchip fixture functioning as the interface of the system to the microchip consists of a chip platform and a manifold, as shown in Figure 3C. The frames of the fixture are made of Teflon, providing excellent chemical resistance and thermal insulation. The microchip assembly is placed into a recessed area on the chip platform and held in place with the manifold using screws. Underneath the glass CAE chip is an aluminum plate with a thin film heater controlled by a commercial PID module (XMT-624-SSR, HBKJ, Beijing, China) for heating the chip during electrophoresis. As shown in Figure 3D, the chip manifold contains two portions: first, above the DEA chips, fluidic microports connecting to the fluidic system, heating blocks for PCR thermal cycling, valve plungers and injection electrodes with self-locking solenoids are installed in the positions corresponding to the structure of the DEA chips. Second, above the CAE chip, platinum electrodes supplying voltages to the corresponding buffer reservoirs are installed in the manifold.

Procedure of "Sample-in-answer-out" genetic analysis

To prepare the CAE microchip, a dynamic coating solution (DEH-100: methanol =1:1, The Gel Company, San Francisco, CA) was first injected into the channels using a syringe and incubated for 1 min, followed by washing with DI water. A sieving matrix, 4.5% (w/v) linear polyacrylamide (LPA) with 6 M urea in 1×Tris TAPS EDTA (TTE), was then loaded into the channels from the anode wells using a custom-made loading machine

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with a driving motor. After extra 1× TTE buffer was pipetted into the PDMS reservoirs, the CAE chip was placed onto the microchip platform and aligned to the detection window. Next, raw samples, such as whole blood, bloodstains and buccal swabs, were loaded into the loading chambers of the DEA chips, which were then sealed with a piece of adhesive tape. Two DEA chips were placed onto the platform in positions where the injection chambers of the chips were aligned to the corresponding sample reservoirs in the CAE microchip. The chip manifold was closed to press the microchip assembly from the top. Due to the natural adherence of PMMA to PDMS, non-leaky sealing between the DEA and the CAE chips was obtained with the simple press by the manifold. Two Eppendorf tubes containing freshly prepared PCR Master Mix were inserted into the manifold and connected to the fluidic control system via PTFE tubing.

Once the preparation procedure is finished, the instrument was turned on and the entire genetic analysis was conducted by the instrument automatically under the control of the LabVIEW program without any human intervention. First, DNA extraction was carried out following a similar protocol developed previously [31, 32]. Briefly, 200-μL distilled water was aspirated through the DEA chip to rinse the raw samples and to bring cells to the downstream filter disc in the reaction chamber, where cells were lyzed completely by 100-μL NaOH (20 mM) within 5 min. After that, diluted HCl (1.8 mM) was loaded into the chip followed by 1×TE buffer. This extensive rinsing step will stabilize the pH value in the reaction chamber. The thermal cycling process was started while all the on-chip valves were closed.

After the thermal cycles were completed, PCR products in the reaction chamber were pumped into the upper compartment of the injection chamber. The injection electrodes with a cross groove were actuated by the

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solenoids to punch through all the sealing tapes of the injection chambers. The amplicons, together with the DNA markers and formamide in the lower compartment, were transferred to the sample reservoirs of the CAE chip. The CE separation was performed after the temperature of the microchip platform reached 60 ^{o}C. The samples were electrophoretically injected toward the waste by applying an electric field of 100 V/cm for 90 s while floating the anode and cathode. A separation field of 180 V/cm was then applied between the cathode and anode to start DNA migration along the 16-cm-long separation channel. The entire "sample-in-answer-out" analysis takes 2 hours to complete without any manual operations. Once the genetic analysis is finished, the whole microchip assembly was taken out from the instrument. Two plastic DEA chips were disposed, while the glass CAE chip was cleaned for the next use.

Singleplex PCR system and sample preparation

A set of primers (forward: TAMRA-5'-CCCTGGGCTCTGTAAAGAA-3', reverse: 5'-ATCAGAGCTTAAA CTGGGAAGCTG-3', Sangon Biotech, Shanghai, China) for amplification of a 106-bp and a 112-bp fragment from the amelogenin gene in X and Y chromosome, respectively, is used for system characterization. The 100-μL Master Mix consists of 10-μL PCR buffer (10×, Roche, Indianapolis, IN), 3-μL dNTP (10 mM each), 8-μL $MgCl_2$ (25 mM), 5-μL Roche FastTaq polymerase, 10-μL bovine serum albumin (BSA, 50 μg/μL, Sigma-Aldrich, St. Louis, MO), 3-μL PEG10000 (160 μg/μL, Sigma-Aldrich), 2 μL of each primer (10 μM each), and 57-μL DI water. Standard K562 genomic DNA (Roche) was employed to test the performance of the sample-in-answer-out microsystem. Human whole blood was collected from a volunteer with informed consent. Dried bloodstains were prepared by pipetting 2-μL blood on a piece of 903® paper. After dried completely, 3-mm-diameter blood discs were punched off and kept at 4 ^{o}C until use. Buccal swab samples were prepared by gently scraping the inside of a volunteer's cheek for 30 s using Whatman's sterile Omni swabs (GE

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Healthcare). The tips of the swabs (about 3 mm in length) were cut off with a razor blade and stored at 4 ^{o}C. The PCR protocol includes an initial activation of polymerases at 95 ^{o}C for 5 min, followed by 35 cycles of 95 ^{o}C for 30 s, 60 ^{o}C for 30 s and 72 ^{o}C for 30 s, and a final extension step for 10 min at 72 ^{o}C.

Multiplex two-color allele specific PCR for warfarin pharmacogenetic testing

Two-color multiplex allele-specific PCR for detecting the *VKORC1* 6853 (-1639G>A, rs9923231), the *VKORC1* 6484 (1173C>T, rs9934438), and the *CYP2C9**3 (1075A>C, rs1057910) SNPs was developed in collaboration with CapitalBio Corporation (Beijing, China). All the primer sequences were provided by CapitalBio. To amplify each locus, the primer set consists of one shared reverse primer and two forward primers which specifically match two variant alleles and are labeled with FAM and TAMRA, respectively. The sizes of all the amplicons can be found in Table 1. Whole blood samples from five donors were kindly provided by CapitalBio with informed consent. The 100-μL Master Mix for multiplex allele-specific PCR is composed of 10-μL PCR buffer (10×, Roche), 3-μL dNTP (10 mM each), 8-μL $MgCl_2$ (25 mM), 5-μL Roche FastTaq polymerase (Roche), 10-μL BSA (50 μg/μL), 3-μL PEG10000 (160 μg/μL), 22 μL of primer mix and 39-μL DI water. The thermal cycling protocol is the same as that in the singleplex amplification.

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Results and Discussion

Integration of DNA extraction, PCR and CE

Although the translation of individual analytical steps into chip formats, such as DNA extraction, PCR, and capillary electrophoresis, has been extensively published, the development of fully integrated microsystems for "sample-in-answer-out" genetic analysis is still a daunting task. It is because the integration is much more than the simple combination of several stand-alone function units. Efficient and reproducible sample/product transport between functional units is the key to forming a seamlessly integrated system that possesses the advantages of high sensitivity, reproducibility, and reliability. Here we successfully incorporated several key technologies to achieve the goal of "sample-in-answer-out". First, the integration of DNA extraction and PCR amplification is realized by employing an "*in-situ*" amplification concept.[31, 32] In contrast to the most common bind-wash-elute DNA extraction,[34, 35] the "*in-situ*" amplification combines the DNA extraction and PCR in a single reaction chamber, where amplification is performed directly without elution. This method not only dramatically simplifies the structures of the integrated system, but also ensures the entire DNA extract being mixed with PCR reagents without timing issue, leading to more sensitive detection.

Second, a novel injection electrode coupled with an injection chamber was developed for the integration of the sample preparation and the detection units. This new mechanism has three functions: 1) acting as a valve to isolate the PCR and the CE structures; 2) mixing PCR products with sizing standards and formamide; 3) transferring the mixture from the DEA chip to the CAE chip. A piece of tape on the bottom of the injection chamber functions as a valve to isolate the sample preparation and the capillary electrophoresis chip in this system. To open this "tape valve", the electrode is actuated to penetrate all the way through the DEA chip so that the tip of the electrode contacts the bottom of the sample reservoir of the CAE chip. Due to the capillary

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action, PCR products and the injection reagents stored in the injection chamber are drawn into the cross groove of the electrode, where the solutions are thoroughly mixed together. After that, an electrical field is applied between the electrode and the waste reservoir to electrophorese the mixture from the groove to the injection channel for analysis. To verify the functions of this mechanism, we first loaded blue and red ink into the upper and the lower compartments of the injection chamber, respectively (shown in Figure 4A). The electrode was manually punched through the injection chamber and then immediately taken out to dispense all the absorbed solution into a serial of spots on a piece of paper. As shown in Figure 4B, all the spots show a uniform purple color, illustrating that the blue and red ink were mixed very well in the groove. To further verify the electrical injection of this mechanism, we conducted the electrophoretic separations of the samples that was injected conventionally and by the mechanism, respectively. Both injection methods can produce similar electropherograms (Figure 4C) with balanced peak heights in the range of 100-300 bp, demonstrating the effectiveness of the injections. This simple integration and injection method further simplified the structures of the microchip assembly.

Third, we adopted a modular-based integration approach to construct our "sample-in-answer-out" microsystem. This strategy provides two key advantages to our system: versatility and low cost. Different DEA chips for accommodating different types of samples can all be combined with the same CAE chips via the same integration mechanism. As a result, the assembled microchips are capable of processing multiple sample types, such as whole blood, bloodstains, and buccal swabs, without any changes to the major structures of the instrument. In addition, only the low-cost, plastic DEA chips in the microchip assembly are for one-time use, while the glass CAE chip can be used for multiple times. Thus, the cost of the genetic analysis could be significantly reduced. Overall, by employing the above three strategies, diverse samples can be efficiently

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processed and transferred in the microsystem without any complicated structures and delicate operations.

System characterization using singleplex PCR

We first employed standard K562 genomic DNA to verify the integration of the DNA extraction and amplification chip and the capillary array electrophoresis chip. Premixed PCR solutions containing 0.625, 1.25, 2.5, and 5 ng K562 DNA were loaded into the DEA chips and then analyzed in the instrument. Following 35 cycles of PCR amplification, the amplicons were co-injected with ROX-labeled sizing standards into the CE channels for detection using the on-chip injection electrode. As demonstrated in Figure 5A, the 106-bp fragments amplified from the amelogenin marker can be successfully obtained with as low as 0.625 ng input DNA. Multiple primer-dimer peaks were also generated without any interference to the assay. In parallel, negative control containing DI water instead of genomic DNA shows no peaks in the profile, demonstrating the operations were contamination-free. Next, to verify the complete integration of the entire genetic analysis from DNA extraction to CE detection, human whole blood samples were processed and analyzed in the system. 0.3, 0.5, and 1 μL of blood samples were pipetted onto the filter nets in the loading chambers of the DEA chips, and then the chambers were sealed completely with pieces of tape. After the microchip assembly was loaded into the instrument, the automated process was conducted by the system. Figure 5B reveals that, even with only 0.3-μL input blood, the 106-bp amplified fragments can still be successfully obtained together with co-injected sizing standards. The complete procedure for analyzing human whole blood takes 2 hours, including 15 min for DNA extraction, 80 min for PCR, and 25 min for electrophoresis.

Pharmacogenetic testing for guiding warfarin dosage

Pharmacogenetic testing is one of the essential methodologies for implementing the concept of precision

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medicine.[2] However, the current genotyping is still expensive, labor-intensive, and requires dedicated laboratories. Our "sample-in-answer-out" platform with the advantages of short turnaround-time, low cost, and complete automation may provide a promising tool towards the integration of genetic testing in routine clinical diagnosis. Hence, to demonstrate and verify the application of our system, we developed a two-color multiplex allele-specific PCR assay for detecting warfarin-related single nucleotide polymorphisms in collaboration with CapitalBio Corporation. Three SNP loci, including the *VKORC1* 6853 (-1639G>A), the *VKORC1* 6484 (1173C>T), and the *CYP2C9**3 (1075A>C) were chosen due to their significant impact to warfarin dosing algorithms. First, the limit of detection of the system for this three-plex genetic analysis was explored using human blood that carries wild-type alleles. Different amounts of whole blood were loaded into the microchip, and then the automated analyses were conducted by the instrument following the same protocol as that in the singleplex test. Figure 6 shows that a full profile containing three FAM-labeled wild-type amplicons (152 bp at *VKORC1* 6484, 182 bp at *CYP2C9**3 (1075A>C), and 234 bp at *VKORC1* 6583) can be successfully obtained with as low as 0.5-μL input blood. A blank profile in the negative control confirms that the contamination-free operations were achieved.

Following the limit-of-detection analysis, we then employed blood samples obtained from volunteers carrying various SNP genotypes to test the typing accuracy of the microsystem. In each assay, 2-μL whole blood was processed by the instrument automatically. The entire analytical process including DNA extraction, amplification, and CE detection was performed under the control of the LabVIEW program within two hours. Figure 7 panel A presents a profile obtained from a control sample without any mutations, showing three FAM-labeled peaks. Panel B shows the result from a patient carrying homozygous mutants in all three loci, which produced three TAMRA-labeled peaks, including 150 bp at *VKORC1* 6484 (1173 T>C), 183 bp at

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*CYP2C9**3 (1075A>C), and 234 bp at *VKORC1* 6583 (-1639G>A). Due to the color change of the PCR products, the genotyping results can be read out immediately. Panel C is the genotyping from a sample with heterozygous alleles in the *CYP2C9**3 SNP locus. Two peaks, one labeled with FAM from the wild-type allele and the other labeled with TAMRA from the mutant allele, were generated. Since the electrophoresis performed in our system can achieve a single-base separation resolution, the size and the color differences in these two peaks clearly illustrate the genotype of the blood sample. Next, the electrophoregram shown in Panel D contains a 150-bp TAMRA peak, a 182-bp FAM peak, and a 234-bp TAMRA peak, illustrating that the blood carrying two homozygous SNPs at *VKORC1* 6484 and *VKORC1* 6583. Finally, the sample with two heterozygous SNPs at *VKORC1* 6484 and *CYP2C9**3 was analyzed, producing two peaks in each locus (Panel E). All of the genetic profiles obtained from the fully integrated and automated system were in 100% accordance with those using conventional methods, demonstrating our microsystem has the capability of detecting SNPs correctly in a timely manner.

Genetic testing from bloodstains and oral swabs

Since multiple types of samples may be encountered in clinical diagnosis, we further evaluated our system's versatility using dried bloodstains and oral swabs. To analyze samples with carrying materials, the DEA chip in the microchip assembly was swapped to the one that has an empty loading chamber without the filter net. In the bloodstain test, a 3-mm-diameter disc of Whatman 903® paper that contains 2-μL human whole blood was sealed into the loading chamber with a piece of tape. Once the chips were assembled and put into the instrument, the automated analytical process was conducted by the instrument following a similar protocol to that in the whole blood analysis. The electrophoregram showing three FAM-labeled peaks at the *VKORC1* 6484, the *CYP2C9**3, and the *VKORC1* 6583 loci was listed in Figure 8A. To type oral swab samples using

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our instrument, the tip (about 3 mm in length) of a Whatman's sterile Omni swab was cut off with a razor blade and then put into the loading chamber of the microchip assembly. After that, the rest of the analysis is the same as that of bloodstains. Figure 8B shows that a full profile with three FAM-labeled peaks was successfully obtained. These results clearly indicate that the fully integrated microsystem is capable of conducting automated genetic analysis from multiple sample types with a simple modifications to the disposable DEA microchip.

The conventional genotyping methods based on the allele-specific PCR assay usually rely on several bulky instruments, have a lengthy process, and are prone to contamination. For examples, the procedure of the microarray-based method includes DNA extraction, PCR, single-strand DNA preparation, hybridization, washing, and detection, which takes about 7-8 hours to complete, and needs a lot of manual operations. Real-time PCR detection is much faster and the operation is relatively simple, but the typing accuracy is often not adequate for clinical diagnosis. In contrast, our microfluidic system integrates the entire procedure of a genetic testing into a single modular-based microdevice and the total analysis time was reduced to two hours. As a result, the sample handing by users in our system is significantly reduced, eliminating the risk of cross-contamination and simplifying the operations. The electrophoretic separation reveals the sizing information of the PCR products, providing an additional check of the typing accuracy. In the future, it is possible to extend the use of our system to more directions, such as point-of-care diagnosis, forensic human identification, food safety testing, and environmental monitoring.

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Conclusions

The fully integrated "sample-in-answer-out" microsystem for pharmacogenetic analysis successfully combines microchip sample preparation and capillary electrophoresis into a modular-based microdevice operated in a compact control and detection instrument. The "*in-situ*" PCR amplification enabled by the filter paper-based DNA extraction method dramatically simplifies the structure of the microchip. In addition, by employing a novel injection electrode, the plastic DEA chips were integrated with the glass CAE chip in a modular format, which facilitate the analysis of various sample types. The sensitivity of the microsystem was determined to be 0.625 ng K562 standard DNA or 0.3 μL human whole blood for the singleplex detection of the amelogenin marker, and 0.5 μL blood for the genotyping of three warfarin-related SNPs. The entire genetic analysis from a sample to a profile can be completed in two hours without any human intervention. The successful development of this fully integrated and automated microsystem is a significant advance towards rapid, sensitive, and reliable pharmacogenotyping for guiding patients' warfarin dosing in clinical practice. In the future, since the extraction-amplification-electrophoresis process is a common protocol for genetic analysis, our instrument can be extensively used for many purposes, such as forensic DNA typing, food safety, environmental monitoring, etc., without any major modifications to the system.

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Acknowledgements

We thank Prof. Jing Cheng, Prof. Wanli Xing, and Prof. Guoliang Hua at the Department of Biomedical Engineering, Tsinghua University for their valuable discussions. This research was supported by National Instrumentation Program (No. 2013YQ190467) from Ministry of Science and Technology of China.

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Tables

Table 1. The sizes of the amplicons in the multiplex allele-specific PCR

	VKORC1 6484 (1173T>C)	*CYP2C9**3 (1075A>C)	*VKORC1* 6583 (-1639G>A)
Wild type (FAM)	152	182	234
Mutant (TAMRA)	150	183	234

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Figure legends

Figure 1. Schematic of fully integrated microchip assembly for warfarin pharmacogenetic testing. (A) Disposable plastic microchip for DNA extraction and PCR amplification. Left: the microchip contains two DNA extraction and amplification units, each of which contains an extraction inlet, a shared PCR inlet, a waste outlet, a loading chamber, a reaction chamber, an injection chamber, and three microvalves. Right: exploded view of the microchip structure. (B) Layout of the 4-inch glass capillary array electrophoresis chip. Upper expanded view shows the structure of the injection channels. Lower expanded view is a tapered turn incorporated into the separation channel. S: sample well (blue), C: cathode well (yellow), W: waste well (black), and A: anode well (red). (C) Photograph of the microchip assembly.

Figure 2. Schematic of injection electrode coupled with injection chamber. (A) The injection electrode with a cross groove is positioned on the top of the injection chamber, which is divided into two compartments: the upper for PCR products and the lower for sizing standards and formamide. (B) The electrode is actuated to penetrate through the injection chamber for injection. The insert is a photograph of the injection electrode.

Figure 3. Photographs of the compact control and detection instrument. (A) Image of the instrument. (B) The fluidic control system installed in the front of the system. (C) The microchip fixture located on the top of the instrument for accommodating the microchip assembly. (D) The structure of the manifold.

Figure 4. Verification of the injection electrode for sample mixing and injection to electrophoresis. (A) Side view of the injection chamber in the DNA extraction and amplification chip. The upper and the lower

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compartment of the loading chamber were filled with blue and red ink, respectively. (B) Colors of the blue, red, and mixed ink on a piece of paper. The ink in the loading chamber were absorbed into the electrode and then dispensed into \a serial of spots on a piece of paper. All the spots in the dotted box show a uniform purple color, illustrating that the blue and red ink were mixed immediately in the groove. (C) Comparison of the electropherograms generated by the conventional and the injection electrode method.

Figure 5. Limit of detection of the microsystem for the amelogenin marker. (A) PCR amplifications and separations performed from K562 standard genomic DNA on the system. The detection limit was determined to be 0.625 ng of input DNA templates. (B) Genetic analyses of human whole blood from DNA extraction to electrophoretic detection. A complete trace was obtained from 0.3-μL blood.

Figure 6. Detection limit of the microsystem for analyzing three SNP loci in *VKORC1* and *CYP2C9*. A full profile was obtained from as low as 0.5 μL of input whole blood. A negative control experiment was conducted to confirm the absence of carryover.

Figure7. Three-plex pharmacogenetic analyses of patients's blood samples with various genotypes. (A) Analysis conducted from 2-μL control sample, showing three FAM-labeled peaks (152 bp at *VKORC1* 6484, 182 bp at *CYP2C9**3, and 234 bp at *VKORC1* 6583). (B) Genetic analysis of a patient carrying homozygous mutations in all three loci, which produced three TAMRA-labeled peaks (150 bp at *VKORC1* 6484, 183 bp at *CYP2C9**3, and 234 bp at *VKORC1* 6583). (C) Analysis of a blood sample with a heterozygous mutant at *CYP2C9**3. One peak labeled with FAM from the wild-type allele and the other labeled with TAMRA from the mutant were

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shown in the trace. (D) Testing of a blood sample obtained from a patient carrying two homozygous mutants in the 6484 and the 6583 SNP loci of the *VKORC1* gene. (E) Detection of a sample with two heterozygous mutations at *VKORC1* 6484 and *CYP2C9**3, showing two peaks in each heterozygous SNP locus.

Figure 8. Pharmacogenetic analyses conducted from (A) a dried bloodstain and (B) a buccal swab. Full profiles could be automatically obtained in two hours by the microsystem.

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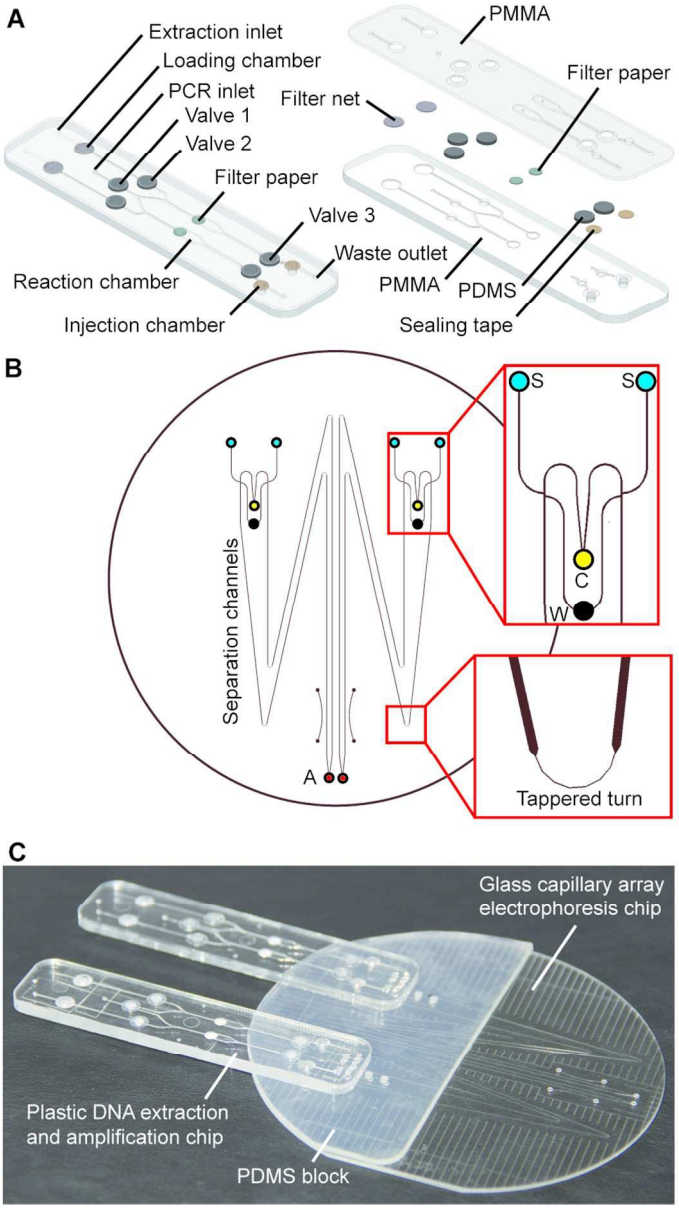

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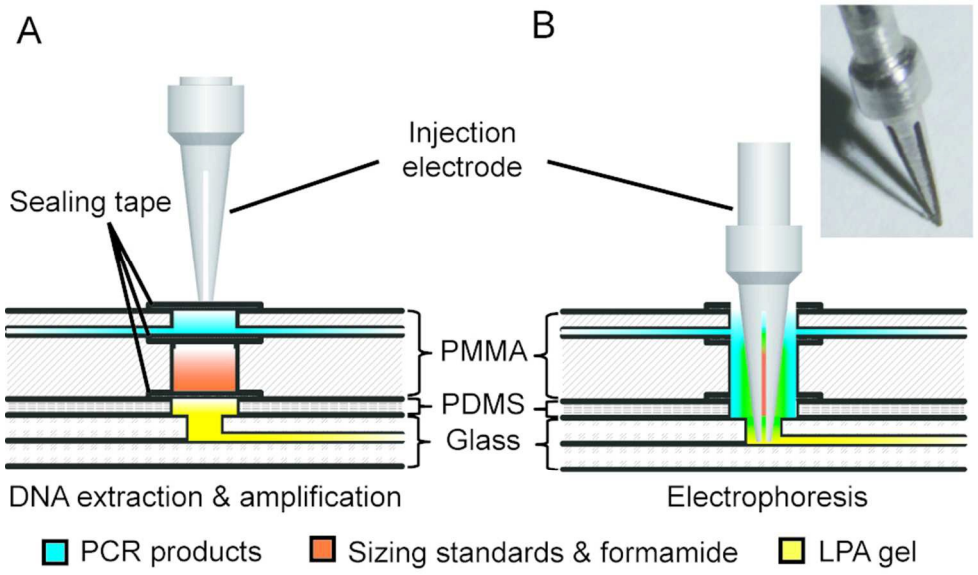

Figure 2
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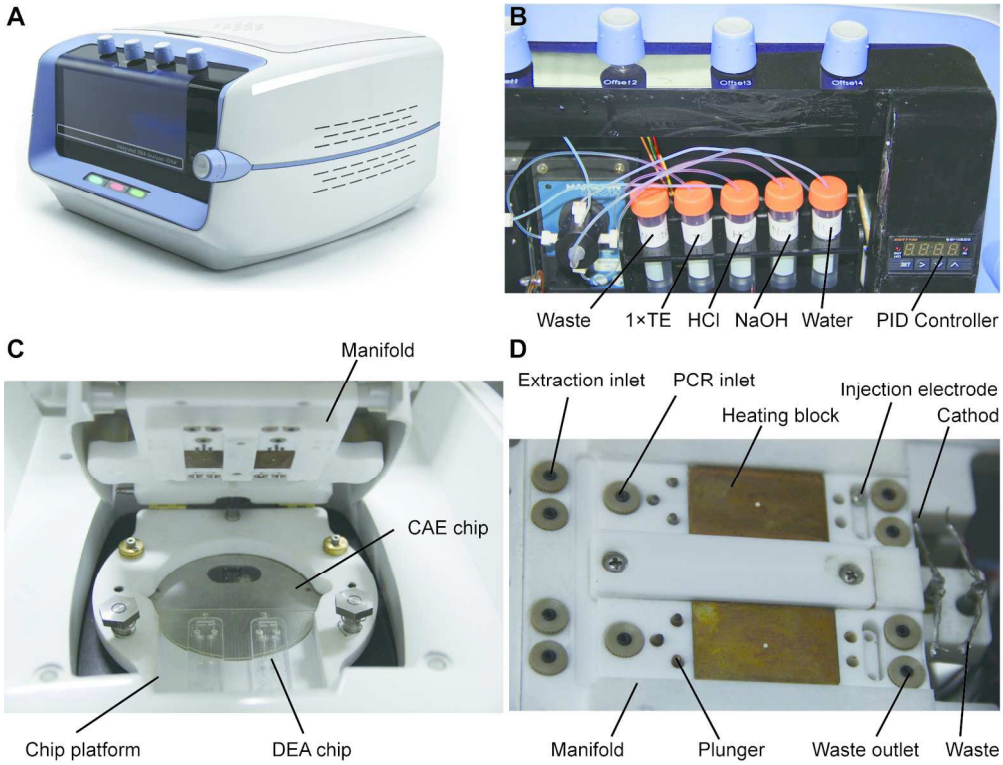

Figure 3
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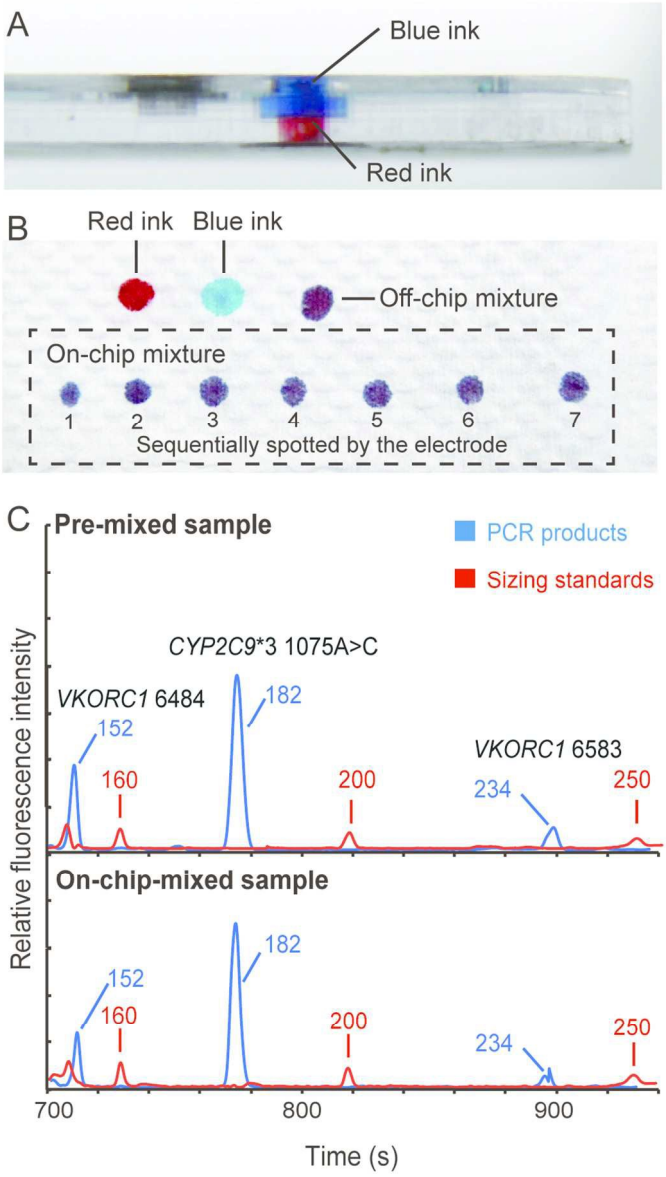

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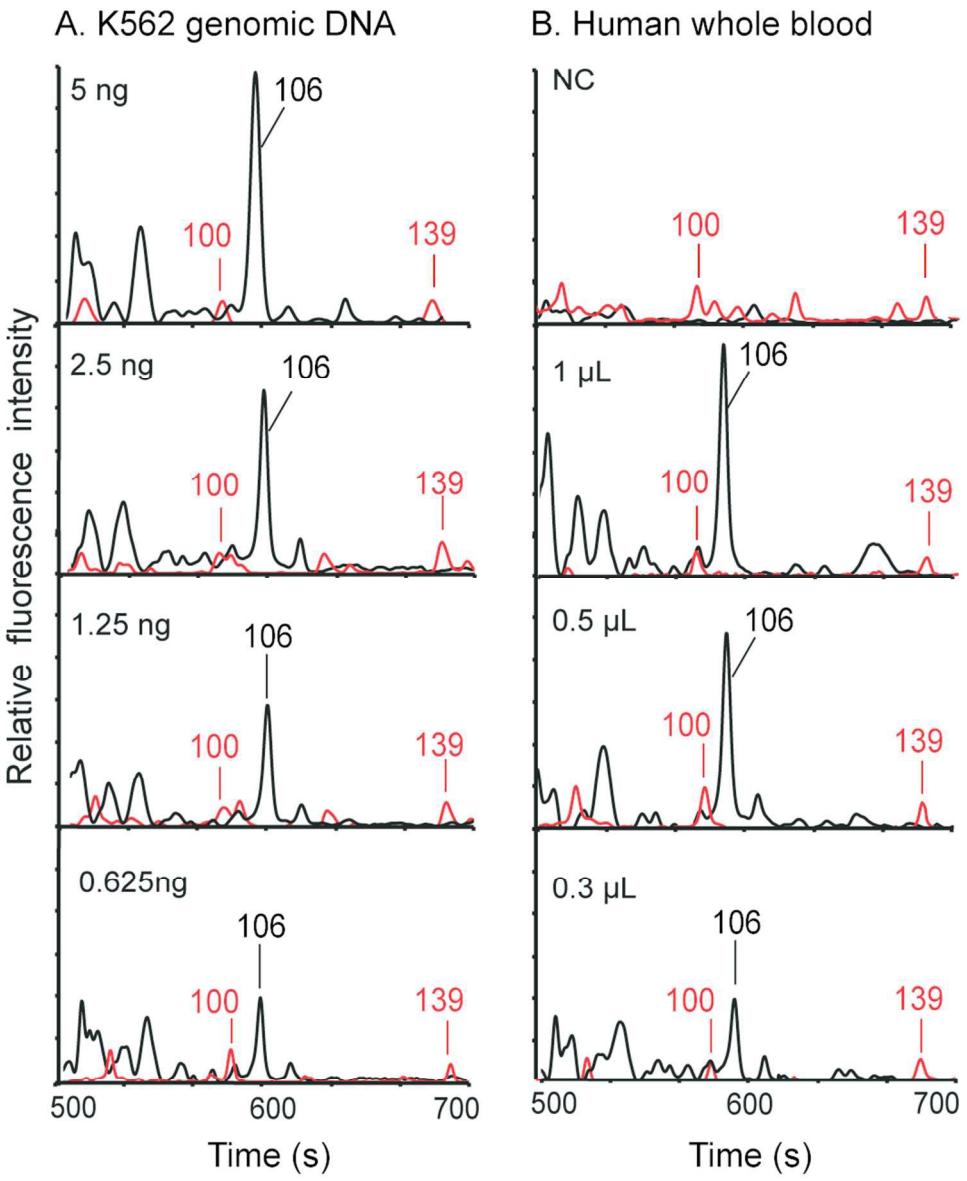

Figure 5
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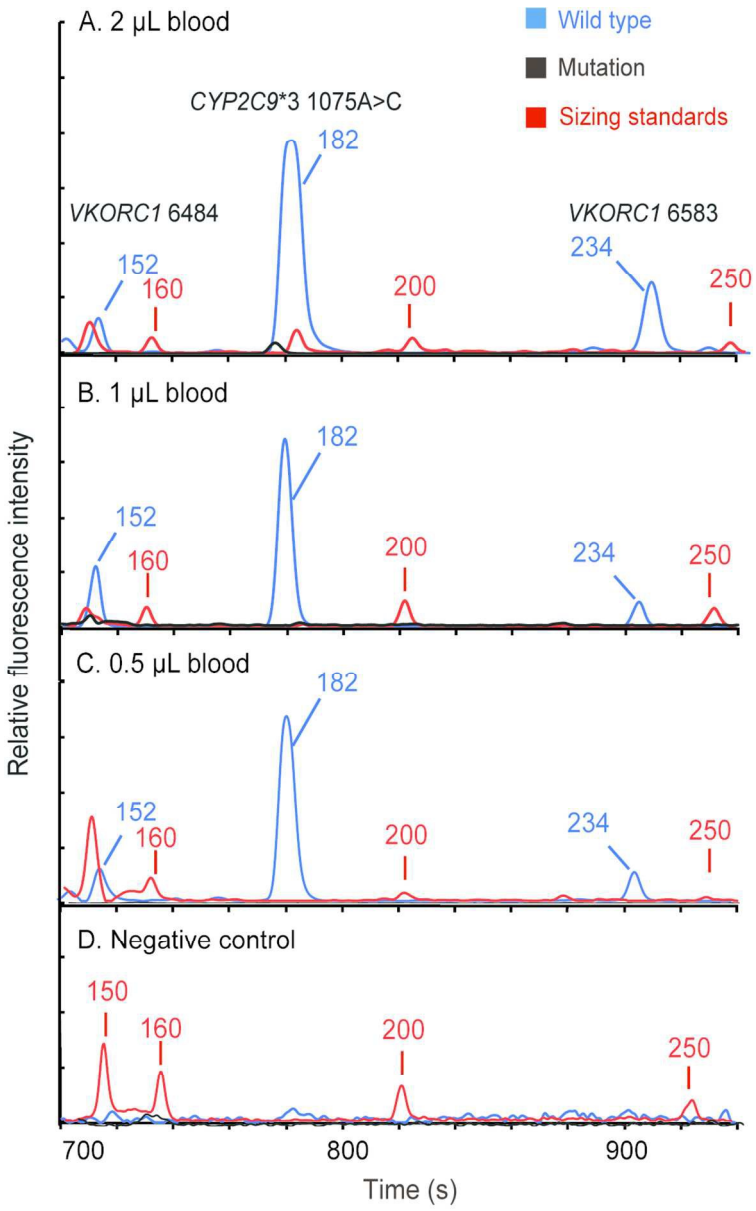

Figure 6
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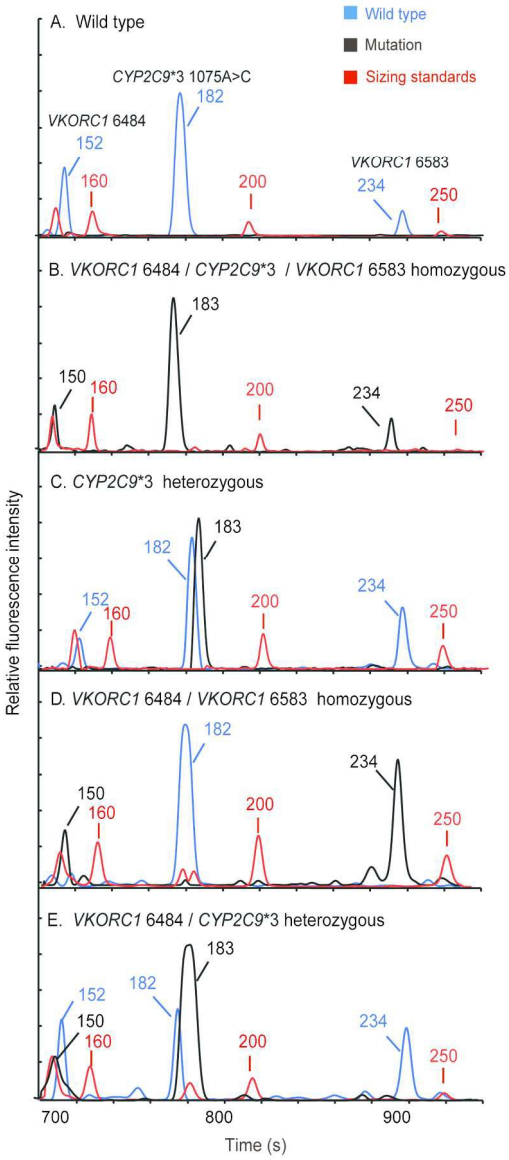

Figure 7
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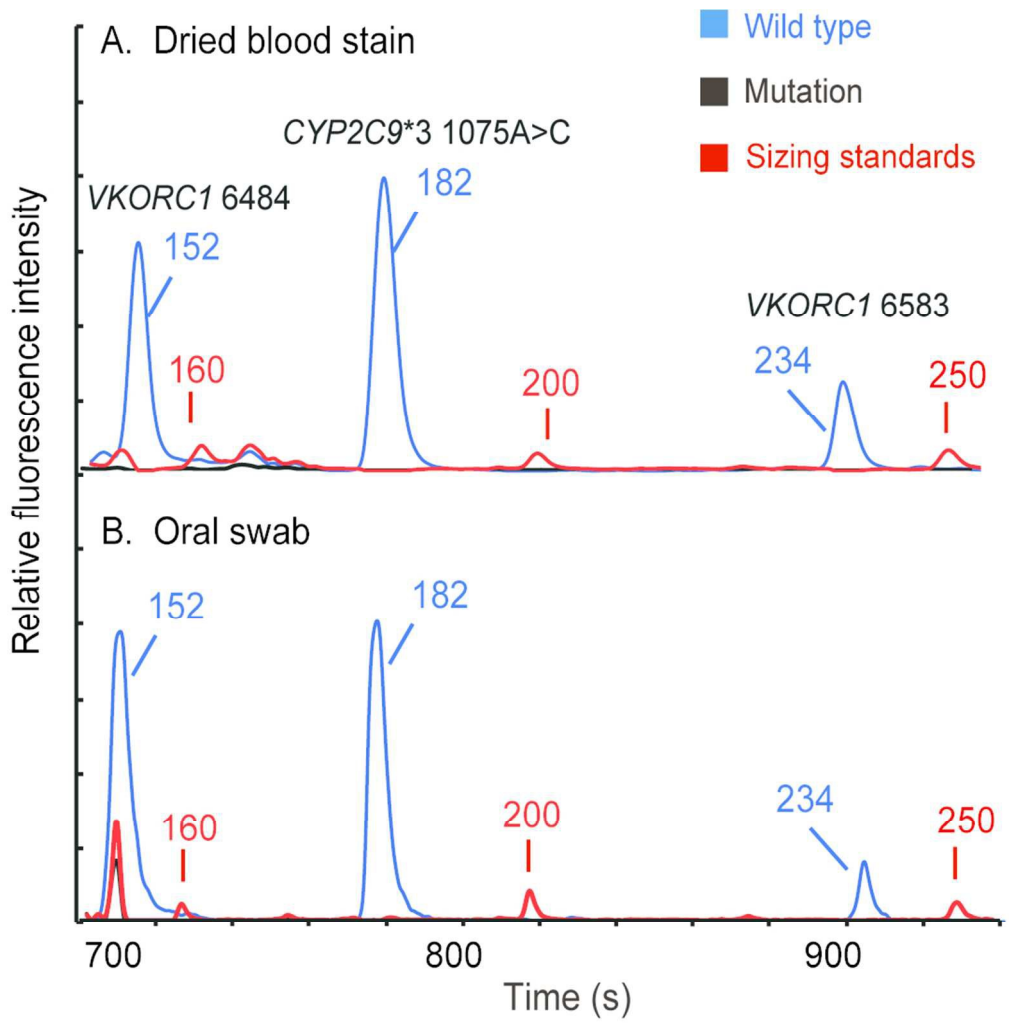

Figure 8
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