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A Filter Paper-Based Microdevice for Low-Cost, Rapid, and Automated DNA Extraction and Amplification from Diverse Sample Types

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Abstract:

A plastic microfluidic device that integrates a filter disc as a DNA capture phase was successfully developed for low-cost, rapid and automated DNA extraction and PCR amplification from various raw samples. The microdevice is constructed by sandwiching a piece of Fusion 5 filter, as well as a PDMS (polydimethylsiloxane) membrane, between two PMMA (poly(methyl methacrylate)) layers. An automated DNA extraction from 1-μL human whole blood can be finished on the chip in 7 minutes by sequentially aspirating NaOH, HCl, and water through the filter. The filter disc containing extracted DNA was then taken out directly for PCR. On-chip DNA purifications from 0.25-1 μL human whole blood yielded 8.1-21.8 ng DNA, higher than those using QIAamp® DNA Micro kits. To realize DNA extractions from raw samples, an additional sample loading chamber containing a filter net with an 80-μm mesh size were designed in front of the extraction chamber for accommodating sample materials. Real-world samples, including whole blood, dried blood stains on Whatman 903® paper, dried blood stains on FTA™ card, buccal swabs, saliva, and cigarette butts can all be processed in the system in 8 minutes. In addition, a multiplex amplification of 15 STR (short tandem repeat) loci and a Sanger-based DNA sequencing of the 520-bp GJB2 gene were accomplished from the filters that contain extracted DNA from blood. To further prove the feasibility of integrating this extraction method with downstream analyses, "*in-situ*" PCR amplifications were successfully performed in the DNA extraction chamber following DNA purifications from blood and blood stains without DNA elution. Using a modified protocol to bond the PDMS and PMMA, our plastic-PDMS devices withstood the PCR process without any leakage. This study represents a significant step towards the practical application of on-chip DNA extraction methods, as well as the development of fully integrated genetic analytical systems.

Key words:

Solid-phase DNA extraction, Microfluidics, On-chip PCR, Plastic-PDMS bonding, Short Tandem Repeat

1. Introduction

Genetic analysis, such as Sanger-based DNA sequencing, pathogen detection, and short tandem repeat (STR) analysis, has played an increasingly important role in health care, food safety, forensic science, etc [1-3]. The miniaturization and integration of the whole process of a genetic analysis into a micro Total Analysis System (μTAS) can provide numerous advantages over conventional laboratory-based systems, such as automation, reduced cost, and on-site deployment [4, 5]. Although significant progress has been achieved [5-7], microchip-based analytical systems are still primarily utilized by the academic community due to the high cost of the chip microfabrication, complicated operations, and the poor capability of processing various real-world samples. To overcome these challenges, DNA extraction is the one of the major targets that many microfluidic researchers are aiming at [8], because DNA extraction, as the first step in the overall genetic analytical process, determines what types of samples the system can process and how high the quality of DNA template can be provided to the following analytical steps.

Adopting the ideas from the off-chip counterparts, the most commonly used method in the early development of the on-chip DNA extraction technology is based on the capture mechanism of DNA adsorption to silica surfaces in the present of high chaotropic salts [9]. The first on-chip DNA extraction was demonstrated by Christel *et al.* on a microdevice with silica pillars fabricated by deep reactive ion etching (DRIE) for nucleic acid capture [10]. Instead of microfabrication, Breadmore *et al.* from Landers' group successfully created a silica bead-packing column with the aid of a sol-gel matrix in a microdevice for DNA purification, demonstrating near 80% of DNA recovery rate [11]. Magnetic silica beads retained by a magnetic field were also used as a fluidic capture bed in a microchannel for DNA isolation from blood [12, 13]. While silica-based methods are effective, the use of PCR-unfriendly reagents, high-concentration chaotropes and organic solvents, often poses problems to the

downstream amplifications. To circumvent the problem, Cao *et al.* utilized chitosan-coated beads to extract DNA at pH 5 and release it from beads at pH 9, achieving an impressive extraction efficiency of 75% from whole blood samples [14]. Different surface modification methods, such as amine and carbonyl/carboxy group grafting, were also proved to be effective for pH-induced DNA extractions [15, 16]. Unfortunately, the utilization of on-chip DNA extraction methods on a daily base so far is still rare.

In our opinion, an optimal DNA extraction microchip should satisfy the following criteria: (1) providing adequate inhibitor-free DNA to downstream analyses; (2) inexpensive; (3) automated; (4) capable of processing a variety of raw samples; (5) suitable for integration with downstream steps to form a total analytical system. The early development of microchip-based DNA extractions was primarily focused on achieving a high extraction efficiency [17]. Until in recent years, more and more attentions are being shifted to the rest criteria. First, to lower the cost, various polymer substrates, such as poly(methyl methacrylate) (PMMA) [18-20], polycarbonate (PC) [21], and photoactivated polycarbonate (PPC) [16], have been employed to fabricate DNA extraction systems. More recently, a microdevice made of polymer sheets and paper has been developed to enable truly low-cost, point-of-care sample preparations in the developing world [22]. These inexpensive materials not only dramatically simplify the fabrication process, but also eliminate the risk of cross-contamination by enabling one-time use of the devices.

The automation and the capability of processing various raw samples are also the major issues that should be carefully addressed together. The process of the DNA capture and elution on a chip can be easily transformed to an automated procedure with the aid of computer-controlled fluidic systems [8]. However, when dealing with raw samples, such as buccal swabs or dry blood stains, most chip-based systems require lengthy manual

operations to prepare samples. For instance, Liu *et al.* successfully developed a fully integrated system capable of analyzing buccal swab samples [7]. However, before loading samples to the chip, buccal swabs have to be squeezed against a tube wall to release cells into a solution. Many research groups have realized the importance of these issues to the on-chip systems. Haswell's group developed an integrated DNA extraction and amplification device that can directly accept buccal swabs without any sample preparations [23]. Lounsbury *et al.* from the Landers group demonstrated a microsystem that is equipped with a receptacle for accommodating a portion of a buccal swab and whole blood [18]. However, these improvements often sacrifice the simplicity of the systems, and the types of samples which can be processed are limited.

Finally, the challenge than must be addressed is how to make the on-chip DNA extraction suitable for integration with downstream steps in a micro total analysis system. The majority of the on-chip extraction systems employed a bind-wash-elute protocol. In a fully integrated microsystem, this elution step often causes the dilution of extracted DNA, lowering the sensitivity of the genetic analysis [5]. In addition, a good timing is often required in order to load PCR-compatible DNA template into the following PCR chamber [6, 24]. To resolve this problem, a novel "*in-situ*" PCR method performed directly in the same DNA extraction chambers without elution was developed. For examples, a nanoporous aluminium oxide membrane (AOM) was successfully integrated into a microfluidic system for DNA extraction from lysed whole blood and direct PCR [25, 26]. Filter paper, such as FTA™ membrane, was also integrated into a single-chamber cassette for isolation and concentration of DNA, followed by direct LAMP (Loop-mediated, isothermal Amplification) detection [27]. As demonstrated in these studies, the *in-situ* amplification concept simplifies the structures of integrated genetic analytical systems, and improves the sensitivity due to the efficient use of the entire extract.

Although significant advances have been achieved as mentioned above, to the best of our knowledge, no single microdevices have demonstrated the performances that can satisfy all the criteria of an optimal DNA extraction. In 2009, Jangam *et al.* developed a filtration isolation method for HIV proviral DNA extraction from whole blood [28]. In this study, a Fusion 5 membrane disc was employed to capture DNA and an absorbent pad was used for drawing samples through the disc. While the simplicity and the low cost of this device are impressive, this method cannot process diverse raw samples and is not suitable for integrating into a micro total analysis system. Based on the similar DNA extraction mechanism, we further developed a microfluidic device that integrates a Fusion 5 filter disc as a solid capture phase for low-cost, rapid and automated DNA extraction and PCR amplification from a variety of raw samples. The DNA capture efficiency of the microdevice was examined using human whole blood and compared with those using commercially available QIAamp® DNA Micro kits. The capability of the system for processing real-world samples were thoroughly tested using a variety of raw samples. To further examine the DNA quality provided by the on-chip extraction, a multiplex amplification of 15 short tandem repeat (STR) loci and a Sanger-based DNA sequencing of the 520-bp GJB2 gene using the extracted DNA from whole blood were performed. Finally, on-chip PCR amplifications following DNA purifications on the device were conducted in the DNA extraction chamber without elution. This study is a significant step towards the practical application of the on-chip DNA extraction technique.

Materials and methods

Microdevice design and fabrication

The DNA extraction device for extracting DNA from human whole blood is illustrated in Figure 1A. This device is constructed using a three-layer wafer stack, consisting of (from top to bottom) an upper PMMA layer, a PDMS (polydimethylsiloxane) membrane (BISCO® HT-6240, Rogers Corporation, Woodstock, CT), and a lower PMMA layer. A 3-mm-diameter piece of Fusion 5 filter paper (GE Healthcare, Pittsburgh, PA), as well as the PDMS membrane, is sandwiched between these two PMMA layers. The PDMS membrane is employed as an adhesion film for reversibly bonding the device. On the bottom side of the upper PMMA layer, a microchannel (1 mm wide, 350 μm deep) is fabricated to connect a 1-mm-diameter inlet reservoir to a 2.3-mm-diameter drilling hole. On the top side of the lower PMMA layer, a similar channel (1 mm wide, 700 μm deep) is designed to connect a round chamber (2.3 mm in diameter, 700 μm in depth) to a 1-mm-diameter outlet reservoir formed by cutting through the PDMS membrane and the upper PMMA layer. The top drilling hole, a punched hole on the PDMS layer, the filter disc, and the bottom round chamber are aligned together to form a DNA extraction chamber. As shown in Figure 1C, the filter paper disc is located in a circular lower step (3 mm in outer diameter and 2.3 mm in inner diameter, 200 μm in depth). In the experiment of testing different types of filters, the depth of this lower step is changed according to the thicknesses of the filters. In order to process raw samples, such as dried blood stains and buccal swabs, a modified DNA extraction microdevice was designed as shown in Figure 1B. A sample loading chamber with a similar structure is designed in front of the DNA extraction chamber. The diameter of this additional chamber is 4 mm and the dimensions of the circular lower step is 5 mm in outer diameter, 4 mm in inner diameter, and 100 μm in depth. A 5-mm-diameter hydrophilic nylon net filter with an 80-μm pore size (Millipore, Billerica, MA) was embedded in the chamber for retaining sample materials. These two chambers are sealed with ARseal™ adhesive tapes (Adhesive Research Inc., Glen

Rock, PA) during operations

The device fabrication was performed as follows: the microstructures on the PMMA layers were fabricated using a milling machine equipped with a computer numerical control (Model 5410, Sherline, Vista, CA). After milling, holes were drilled using a table drilling machine. The filter paper discs were punched off using a manual puncher with various punch sizes. Before assembling, all the layers were sequentially cleaned using detergent, deionized water (DI water), and ethanol. After completely dried by N_2, they were carefully assembled in a clean hood. To operate this chip, a custom-made plexiglass manifold (Figure 1D) was employed to provide fluidic connections. A syringe pump (PHD 22/2000, Harvard Apparatus, Holliston, MA) was connected to the outlets of the manifold and a switching valve (MVP, Hamilton, Bonaduz, Switzerland) was connected to the inlets through PTFE tubing. Both the pump and the valve are controlled by a custom-built LabVIEW (NI, Austin, TX) program to facilitate automated operations.

To perform DNA extraction and PCR on the chip, the three-layer wafer stack of the microdevice was permanently bonded together in order to prevent leakage during thermal cycling using a modified protocol [29]. Schematic of the PMMA-PDMS bonding process is shown in Figure 1S. Briefly, the surfaces of the PMMA substrates were activated 120 s with oxygen plasma (30% power) to generate hydroxyl groups. Then, the surfaces were silanized using 2% aqueous solutions of APTES (aminopropyltriethoxysilane) at 55 ^{o}C for 1 hour. The surfaces of the PDMS membrane were also treated with oxygen plasma (30% power) for 35 s and silanized using 2% GPTS (glycidoxypropyltrimethylsilane) solution in isopropyl alcohol at 55 ^{o}C for 1 hour. After thoroughly washed with water and dried with N_2, these three PMMA and PDMS layers were aligned and pressed at 70 ^{o}C with a pressure of 4 bar for 4 hours using a hydraulic compression press (Model 15-1-HT, Grimco,

Paterson, NJ) to form a strong and irreversible PMMA-PDMS-PMMA bonding through covalent bonds.

Optimization of filter paper and pump speeds

Fusion 5 filter paper with an average particle retention size of 2.3 μm was purchased from GE Healthcare and other filter papers, including Whatman Grade 1 (11 μm particle retention size), 2 (8 μm), 3 (6 μm), 5 (2.5 μm) and 602h (< 2 μm), were also from GE Healthcare. All these filters were assembled into the microdevices as shown in Figure 1A. To test the DNA capture efficiency, 10-ng K562 standard DNA (Promega, Madison, WI) in a concentration of 5 ng/μL were pipetted onto the surfaces of the filters and washed with 100 μL of DI water. After that, the filter discs were taken out for the real-time PCR quantification following the protocol listed below. To evaluate the impact of flow speeds to the DNA capture, the microdevice integrated with the Fusion 5 filter disc was employed. Ten-ng K562 DNA were loaded into the device and flushed with 50-μL water at the speed of 5, 10, 20, 50 mL/h using the syringe pump. The filters were then taken out for PCR verification.

PCR verification, gel electrophoresis and real-time PCR quantitation

A set of primers amplifying the Amelogenin marker from human genomic DNA was employed to verify the on-chip DNA extractions. The sequences (Forward: 5′-CCCTGGGCTCTGTAAAGAA, Reverse: 5′-ATCAGAGCTTAAACTGGGAAGCTG) amplify a 106-bp and a 112-bp fragment of the X and Y chromosomes, respectively. The 25-μL PCR mixture prepared for each reaction was comprised of 0.425 μL each primer (10 μM), 12.5 μL 2× PowerTaq PCR MasterMix (BioTeke, Beijing, China), 11.65 μL DI water, and the filter disc containing DNA templates. PCR was performed in a conventional thermal cycler (PTC-200, MJ Research, Waltham, MA). The thermal cycling protocol includes an initial activation of Taq 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. Two-ng K562 standard DNA was employed as a positive control for PCR operations. PCR products together with a DNA Marker DL2000 (Takara Bio Inc., Shiga, Japan) were electrophoretically analyzed on a 2% agarose gel at 150 V.

To quantify DNA trapped in the filters, real-time PCR analyses were performed on a Bio-Rad iQ5 system (Bio-Rad, Hercules, CA) using the same Amelogenin primers. The total PCR volume was 25 μL, containing 0.425 μL each primer, 12.5 μL Power 2×SYBR real-time PCR premixture (BioTeke), 11.65 μL of DI water, and the filter disc. The PCR protocol is the same as that in the PCR verification experiment.

DNA extraction from blood samples

Human whole blood was obtained from a healthy volunteer after informed consent for research and was anticoagulated in evacuated blood collection tubes. To extract DNA using the microdevice shown in Figure 1A, 1-μL whole blood was first pipetted onto the surface of the Fusion 5 disc. The DNA extraction chamber was then sealed with an ARseal^{TM} tape. The extraction process began with a washing step using 200 μL of DI water aspirated through the disc by the syringe pump. After that, 100 μL of a 10-mM sodium hydroxide (NaOH) solution was drawn into the DNA extraction chamber and incubated for 5 minutes to lyse cells completely, followed by a neutralization step using 50 μL diluted hydrochloric acid (HCl, 0.1 mM) and a final washing step using 50-μL DI water. All the reagents mentioned above were automatically switched and aspirated through the filter disc using the MVP valve and the syringe pump at a speed of 20 mL/h. An air column was introduced between each segment of the solutions in order to prevent reagent crossover. The whole process can be finished in 7 minutes. Finally, the microchip was disassembled and the filter disc was taken out for off-chip PCR amplification.

Analysis of DNA extraction efficiency

The efficiency of the on-chip DNA extraction was tested on the Figure 1A microdevice using 0.25, 0.5 and 1 μL of human whole blood samples following the protocol described above. DNA was then quantitated using the real-time PCR method with the aid of a calibration curve generated using serially diluted standard DNA dried on the paper discs. Each data points were repeated three times to generate standard deviations. As controls, conventional DNA extractions using QIAamp® DNA Micro kits from Qiagen (Germantown, MD) were conducted with 0.25, 0.5 and 1 μL of input blood following the manual. DNA samples were quantitated using the real-time PCR with the aid of a calibration curve from standard DNA without filter discs.

DNA extraction from raw samples

To verify the capability of the microsystem for processing various real-world samples, six different types of raw samples, including whole blood, dried blood stains (DBS) on 903® specimen collection paper, DBS on FTA® card, buccal swabs, saliva, and cigarette butts were tested on the microdevices illustrated in Figure 1B. All dried blood stains were home-made by pipetting 2-μL blood on a piece of 903® paper and a FTA® card, respectively. After dried completely, 2-mm-diameter blood discs were punched off using a puncher 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 Healthcare). The tips of the swabs (about 3 mm in length) were cut off with a razor blade and stored at 4 ^{o}C. To collect saliva, an Oragene•DNA collection kit (OG-500, DNA Genotek Inc., Ontario, Canada) was employed for the collection and long-term storage of saliva samples. Following the manufacturer's instruction, saliva was spitted into the collection well by a volunteer and mixed with the Oragene•DNA solution. 500 μL of Oragene•DNA/saliva mixture was then added into the device for

DNA extraction. Cigarette butt samples were prepared by a volunteer following a normal smoking way. Small pieces of the cigarette butts were sliced off using a razor blade for further on-chip analysis

The DNA extraction process performed on the modified microdevice is similar to the whole blood extraction described above. Briefly, samples were loaded into the sample loading chamber and sealed with the ARseal™ tape. The extraction process still began with a water washing step. However, the 200-µL water was driven over the samples back and forth 3 times in order to effectively wash the cells to the DNA extraction chamber. After that, NaOH, HCl, and water were sequentially aspirated through the paper disc at the same conditions as mentioned above. Finally, the paper discs were taken out for PCR verification.

On-chip DNA extraction and amplification

On-chip PCR amplifications were conducted following the DNA extractions from 2-µL human whole blood and blood stains on 903® paper. A 25-µL PCR mixture, consisting of 0.425 µL of each Amelogenin primer, 12.5 µL MasterMix, 10.525 µL DI water, 0.625 µL PEG (160 µg/µL, poly(ethylene glycol), MW 10,000), and 0.5µL BSA (50 µg/µL), were loaded into the permanently bonded microchip using the syringe pump. Then the microdevice was disassembled from the manifold and the inlets and outlets were sealed completely with the ARseal™ tapes. The entire chip was upside down put into a thermal cycler with a flat heating block (Mastercycler Nexus Flat, Eppendorf, Hamburg, Germany). To compensate the temperature difference between the heating surface and the chip, a calibrated thermal cycling protocol was used, including an initial activation at 96 °C for 5 min, followed by 35 cycles of 97 °C for 45 s, 51 °C for 35 s and 72 °C for 30 s, and a final extension step for 10min at 72 °C. After PCR, the tapes were carefully removed and the products were taken out with a pipette for gel electrophoresis.

Results and discussion

Optimization of on-chip DNA extraction

We first optimized the operation process of the DNA purification on the microdevices. To extract DNA from human whole blood using the microdevice shown in Figure 1A, two microliter of blood were first pipetted onto the Fusion 5 filter disc, followed by flushing DI water for initial washing, NaOH for cell lysis, HCl for solution neutralization, and DI water for final washing. The overall extraction process can be finished automatically by operating a syringe pump and a switching valve under the control of a computer in only 7 minutes, including 5-min incubation for cell lysis, and 2-min washing for drawing all the solutions through the device. To extract DNA from other raw samples, the device that has an additional sample loading chamber (shown in Figure 1B) was employed. After the samples were loaded and sealed into the loading chamber, DI water was first flushed over the samples back and forth to wash cells off to the filter paper in the extraction chamber. After that, the exactly same operation was performed, followed by post PCR validation. Due to the prolonged initial washing step, the extraction time is about 8 minutes. This on-chip DNA extraction process is fast and automated using the program-controlled pump and valve. In addition, the use of inexpensive extraction reagents, as well as the cheap plastic and paper materials for chip construction, makes the microdevices truly low cost.

Many parameters may affect the performance of the on-chip DNA extraction. Among them, the selection of filter paper was identified as the most important one that be thoroughly optimized. The mechanism of DNA extraction from blood by a piece of filter paper is believed as follows [28]: cells in blood were first trapped by the filter paper and then lysed by NaOH. Exposed genomic DNA was trapped in the filter paper due to the entanglement of long DNA molecules with the fibers of the filter paper, while cell debris, hemoglobin, and other PCR inhibitors were all washed through the paper during the following neutralizing and washing steps by HCl

and DI water. An electron microscopy image of the Whatman FTA™ card with entangled DNA (http://www.whatman.com/FTANucleicAcidCollectionStorageandPurification.aspx) clearly demonstrated that DNA molecules can be indeed trapped in the fiber matrix of the filter paper. We compared the DNA capture capabilities of several different types of filter papers, including Fusion 5 and Whatman filter papers (Grade 1, 2, 3, 5 and 602h), with particle retention sizes ranging from less than 2 μm to 11 μm. Ten-ng K562 standard DNA were pipetted onto the filters and washed with water, followed by real-time PCR quantifications. As shown in Figure 2A, the real-time PCR results demonstrated that the Fusion 5 filter paper provided the highest DNA capture efficiency. This result illustrated that the average particle retention size of the filter paper is related to the DNA capture efficiency. We inferred that a filter with too big pores cannot entangle with DNA molecules efficiently, while one with too small pores will trap more PCR inhibitors. In addition, the capture efficiency is also affected by the compositions of the filters, as the Fusion 5 is quite different from the other cellulose-based filters. Other reasons that make the Fusion 5 filter the best choice include its hydrophilic property, a fast wicking speed, and the high compatibility with PCR amplifications.

Flow speed is another parameter that may affect the DNA capture efficiency. Although a high flow speed is preferred due to the shorter process time, it may also produce lower DNA capture efficiency since DNA can be washed off. We tested different flow rates from 5 up to 50 mL/h. As shown in Figure 2B of the gel electrophoresis result, surprisingly, no significant difference among different flow rates was observed. It is partially due to the efficient entanglement of DNA molecules with the fiber network of the paper. Therefore, a pumping speed of 20 mL/h is selected with the consideration of the extraction speed and the ease of operation.

DNA extraction from whole blood and evaluation of capture efficiency

We evaluated the DNA extraction capability of our microdevice using human whole blood. Blood was chosen because it is one of the most difficult and typical biological samples that are often encountered in clinical diagnosis and forensic DNA typing. One-microliter blood was pipetted onto the Fusion 5 filter paper disc, and then cell lysis and DNA capture were performed on the microdevice automatically without any human intervention. The filter discs were then taken out and directly put into Eppendorf tubes for PCR amplifications. As shown in Figure 3A, the 106-bp PCR products were repeatedly amplified out from the filter discs that contain DNA template extracted from whole blood on the microdevice. In contract to the successful amplifications, a direct PCR amplification from a piece of filter paper that contains the same one-microliter blood without on-chip extraction failed to yield any products, demonstrating the effectiveness of the on-chip DNA extraction and the severe inhibition effect of whole blood to PCR. These results clearly illustrate that the on-chip DNA extraction can effectively clear out most PCR inhibitors and provide PCR-ready DNA template for downstream analyses.

The DNA extraction efficiency of the microdevice was evaluated using the real-time PCR quantification method with human blood samples and further compared with those of conventional extractions using a QIAamp® DNA Micro kit from Qiagen. One of the advantages of the on-chip DNA extraction technology stated by previous literatures is that, unlike the conventional, a microdevice can handle minute amounts of samples [8]. Therefore, we chose 0.25, 0.5 and 1 μL of blood as testing samples to compare capture efficiencies between these two methods, even though the minimal input blood for the QIAamp® kit is 1 μL. As shown in Figure 3B, the on-chip DNA extractions successfully produced 5.6±0.6, 10.3±1.8, and 21.8±2.3 ng DNA from 0.25, 0.5 and 1 μL blood. To the best of our knowledge, these extraction efficiencies from blood on the chips are equal or close to many of the previous works [14, 26, 30]. In contrast, the conventional extractions using the QIAamp® kit can only provide 3.6±0.4, 8.1±1.1 and 13.0±3.1 ng DNA, accordingly. These results demonstrate that our on-chip

extraction method is superior to the conventional in situations where trace samples are often encountered, such as forensic DNA typing. In addition, since the entire DNA extract is trapped in the filter paper and can be used as a whole directly for PCR, the template concentration is much higher than those 0.1-0.23 ng/μL concentrations obtained using the Qiagen kit.

Extractions from various raw samples

As mentioned previously, a DNA extraction microdevice should be able to accept a variety of raw samples directly without any additional manual preparations. Here we selected six different types of samples, including whole blood, blood stains on Whatman 903® paper, blood stains on FTA™ card, buccal swabs, saliva, and cigarette butts, to critically test the extraction capability of our system. Whatman 903® paper is widely used for specimen collections in clinical diagnosis, especially in neonatal blood screening. The excellent quality control of the paper manufacture guarantees consistent sample collections. FTA™ card is another product manufactured by Whatman for blood sample collections especially in forensic DNA analysis. Chemicals pre-dried in FTA™ cards during manufacture can lyse cells in blood, denature proteins and protect nucleic acids from nucleases, oxidative, and UV damage during the long-term storage. However, these chemicals if not cleaned out completely can cause severe issues in amplifications, demanding special attentions in the extraction process. Buccal swabs and saliva are the common samples often collected from individuals for a reference purpose in forensic individual identifications. To ensure consistent collections, Whatman's sterile Omni swabs and Oragene•DNA collection kits were chosen due to their widely applications. Cigarette butt is one of the most common forensic evidence collected at crime scenes. It was chosen to critically test our system because DNA left on the cigarette butts could vary dramatically.

To accommodate raw samples in the microchip, a loading chamber containing a piece of a nylon filter net was designed in front of the DNA extraction chamber. The average pore size of this net is about 80 μm, which will effectively block all the solid materials in samples and let cells move to the second filter paper. As illustrated in Figure 4 of the gel electrophoresis images, successful DNA extractions from blood (Fig. 4A), DBS on 903® paper (Fig. 4B), FTA card samples (Fig. 4C), buccal swabs (Fig. 4D), saliva samples (Fig. 4E), and cigarette butts (Fig. 4F) were all repeatedly achieved. The extraction procedures for these six different types of samples are exactly the same: a portion of raw samples was sealed in the loading chamber and then reagents were sequentially drawn through the device. This easy, fast, and automated extraction for directly processing raw samples further proves the value of this microdevice for real-world applications. While only six types of samples were tested in this study, we believe this two-chamber structure with certain modifications has a great potential of accepting a wide range of sample types, such as solid tissues and liquid samples.

STR typing and DNA sequencing of extracted DNA

On-chip DNA extraction should provide adequate, high-quality template for not only simple singleplex PCR amplifications as demonstrated above, but also more complicated genetic analyses, such as forensic STR analysis and DNA sequencing. A typical STR assay, which involves simultaneous amplifications of more than 15 STR loci in a single tube, often requires high-quality DNA template. To test our system for forensic DNA typing, two microliter of human whole blood was first processed on the microdevice. Then, the filter paper was taken out and a quarter of the piece was cut off for a direct STR amplification. As shown in Figure 5A, balanced and full STR profiles were obtained from this DNA-embedded filter disc using the DNA Typer™ 15 kit. All the alleles were accurately resolved and called. Next, a Sanger DNA sequencing of the GJB2 gene was carried out to further validate the DNA extraction on the microdevice. Again, two microliter of human whole

blood was processed on the microdevice first, followed by the amplification of the GJB2 gene and the standard Sanger sequencing. As shown in Figure 5B, the 520-bp sequences of the GJB2 gene were successfully obtained. Called bases between 90 to 500 bp have an average phred quality value ≥ 20 (accuracy ≥99%).

In both the STR analysis and the DNA sequencing demonstrated above, only a quarter of the final filter disc with entangled DNA template was used for the following PCR amplifications. This is because the microdevice extracted so much DNA from only 2-μL blood that the STR and the sequencing traces would be completely saturated if the whole filter disc was used. These two experiments proved that the DNA extraction from two microliter blood could provide adequate DNA template in terms of quantity and quality for downstream complex genetic analyses.

On-chip DNA extraction and PCR

Since the filter paper can be directly used for PCR as demonstrated above, we envision that PCR amplifications can be conducted in the DNA extraction chamber following DNA purifications. In order to run on-chip PCR, our plastic-PDMS microdevice must be able to withstand the thermal cycling without any leakage. Although several groups have already developed protocols for permanently bonding plastic and PDMS together, by far no PCR amplifications have been demonstrated on such devices yet [29, 31-34]. By employing a high-pressure bonding process, our modified PDMS-PMMA bonding method produced non-bubble, irreversible bonding that is compatible with the on-chip PCR experiment (shown in Figure 2S).

Using the permanently bonded microdevice with a single DNA extraction chamber (Figure 1A), we first performed DNA extractions from 2-μL human whole blood following the same protocol as described previously.

After the extraction, PCR reagents were loaded into the device and successful PCR amplifications were finished, as shown in Figure 6A. After that, dried blood stains containing 2-μL blood on 903® paper were processed using the permanently bonded microdevice with the two-chamber structure (Figure 1B), demonstrating successful on-chip PCR amplifications (shown in Figure 6B). These results validated the feasibility of integrating the DNA extraction and PCR together in a single chamber without a DNA elution step. This "*in-situ*" amplification is attracting, because: i) the single chamber design can dramatically simplify the structures of the integrated system; ii) PCR reagents can be easily loaded into the chamber and thoroughly mixed with DNA template without the timing issue; iii) since the entire DNA extract can be used for amplification, the sensitivity can be improved. Therefore, combined with the other advantages, such as automation, low cost, and versatility, we believe our extraction method will be an ideal candidate for integrating into a micro total analysis system.

Conclusions

In this study, a novel plastic microdevice that integrates a piece of filter paper as a DNA capture phase has been developed for on-chip DNA purification and PCR amplification. Successful DNA extractions from human whole blood, dried blood stains on 903® paper, FTA™ card, buccal swabs, saliva, and cigarette butt samples can all be achieved on the microdevice in less than 8 minutes without any human intervention, yielding adequate inhibitor-free DNA template for complex genetic analyses, such as forensic STR analysis and DNA sequencing. PCR amplifications were also successfully performed on the chip following DNA purifications. In summary, the unique features making the technology outstanding includes: i) rapid production of adequate inhibitor-free DNA to various downstream analyses; ii) low costs of the device and the operation due to the use of filter paper and inexpensive chemicals; iii) fully automated operations; iv) universal extractions of human genomic DNA from various raw samples; v) easy integration of DNA extraction and PCR on a single device with the aid of the PMMA-PDMS bonding method. In the future, the surface of the Fusion 5 filter paper can be grafted with functional groups for even more efficient DNA extractions. The integration of this extraction method with downstream analyses on a single microchip is also on the way.

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Figure Legends:

Figure 1. Schematics of the filter paper-based microdevices for DNA extraction. (A) Exploded view of the microdevice with a single DNA extraction chamber. (B) Exploded view of the microdevice with an additional sample loading chamber. The microfluidic devices consist of an upper PMMA layer, a PDMS membrane for chip bonding, a lower PMMA layer, and a 3-mm-diameter disc of the Fusion 5 filter paper. (C) Expanded view of the filter disc, the lower step and the bottom chamber forming the DNA extraction structure. (D) The setup of the DNA extraction system.

Figure 2. (A) Characterization of DNA capture capabilities of different filter papers using real-time PCR quantitations with standard K562 DNA (error bars indicate standard deviations, n=3). Fusion 5 filter paper provided the lowest C_T value. (B) Gel electrophoresis separation of Amelogenin amplicons from standard DNA washed at different pumping speeds from 5 to 50 mL/h. (Lane M: DL 2000 DNA marker; NC and NC': Negative control; 3~10: on-chip DNA washing at different pumping speeds; PC: Positive control from standard K562 DNA.)

Figure 3. On-chip DNA extraction from human whole blood and comparison with the conventional extraction method. (A) Electropherograms of Amelogenin PCR products amplified from 1-μL human whole blood extracted on the microdevice. (Lane M: DL 2000 DNA marker; NC and NC': Negative control; DB: Direct amplification from blood dried on a filter disc without extraction; 3~6: Amplifications from four blood samples extracted on the device; PC: Positive control from standard K562 DNA.) (B) Comparison of DNA extraction yields between the on-chip DNA extraction method and the conventional DNA purification using QIAamp® DNA Micro kits. With 0.25, 0.5, and 1 μL of input blood, on-chip extractions provided 5.6, 10.3, and 21.8 ng

DNA, which are higher than those obtained by the conventional method (3.6, 8.1 and 13.0 ng).

Figure 4. Electropherograms of Amelogenin PCR products amplified from six different types of raw samples: (A) human whole blood, (B) dried blood stains on Whatman 903® paper, (C) dried blood stains on FTA™ card, (D) buccal swabs, (E) saliva, and (F) cigarette butts. Reproducible amplifications were successfully obtained from all these samples extracted on the device using the same automated operation process. (Lane M: DL 2000 DNA marker; NC and NC': Negative control; DB: Direct amplification from blood without extraction; 3~6: Amplifications from raw samples extracted on the device; PC: Positive control from standard K562 DNA.)

Figure 5. Forensic STR Typing and Sanger-based DNA Sequencing from DNA extracted on the microchips. (A) DNA Typer™ 15 STR profiles of DNA extracted from whole blood using the filter paper-based extraction device; (B) Base-called four-color sequencing data of the GJB2 gene fragment amplified from whole blood processed using the device.

Figure 6. Electropherograms of Amelogenin PCR products extracted and amplified from (A) 2-μL human whole blood on the Figure 1A microdevice and (B) dried blood stains on Whatman 903® paper on the Figure 1B microdevice. DNA extractions and PCR amplifications were both successfully performed on the chips. (Lane M: DL 2000 DNA marker; NC and NC': Negative control; 3, 4: Extractions and amplifications on the device; PC: Positive control from standard K562 DNA; DB: Direct amplification from blood without extraction.)

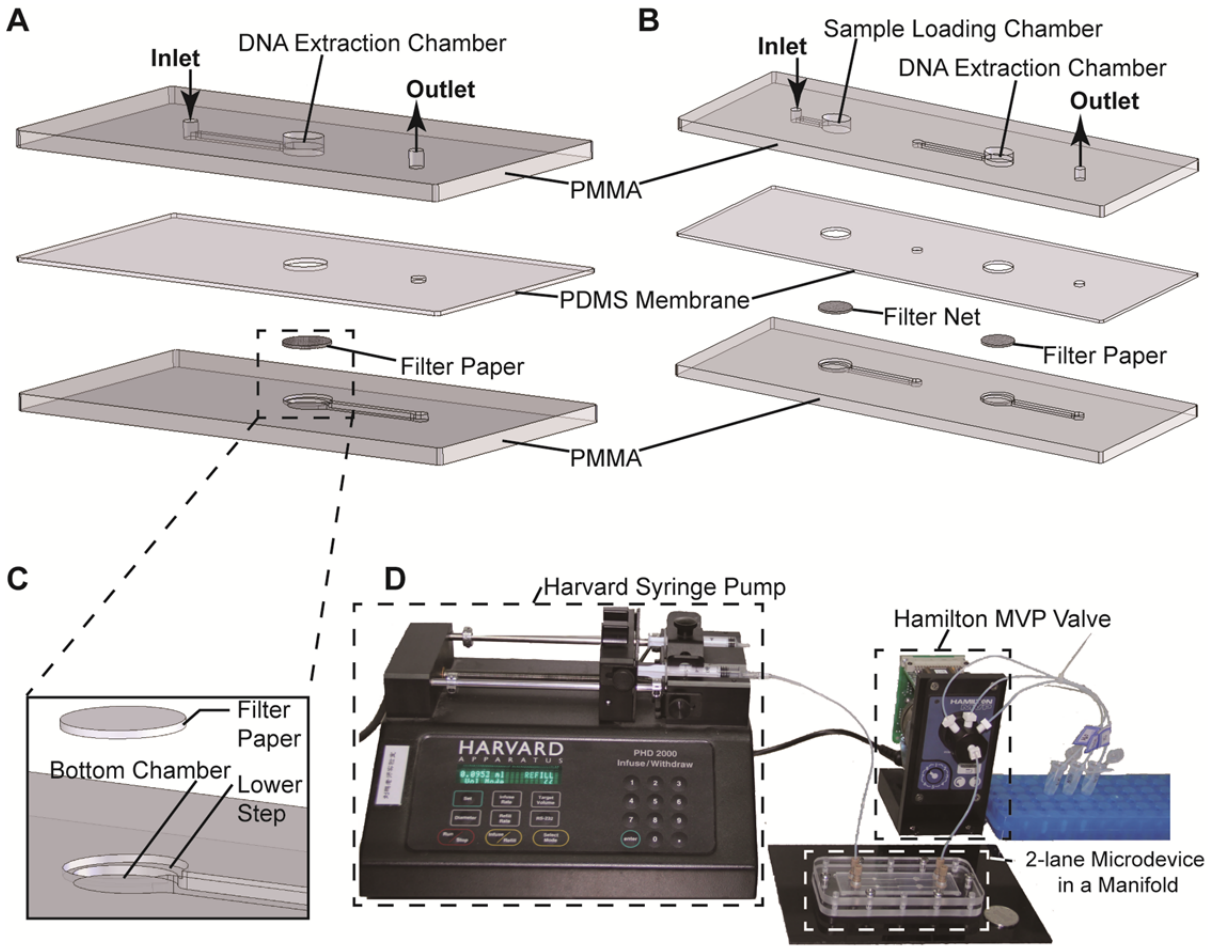
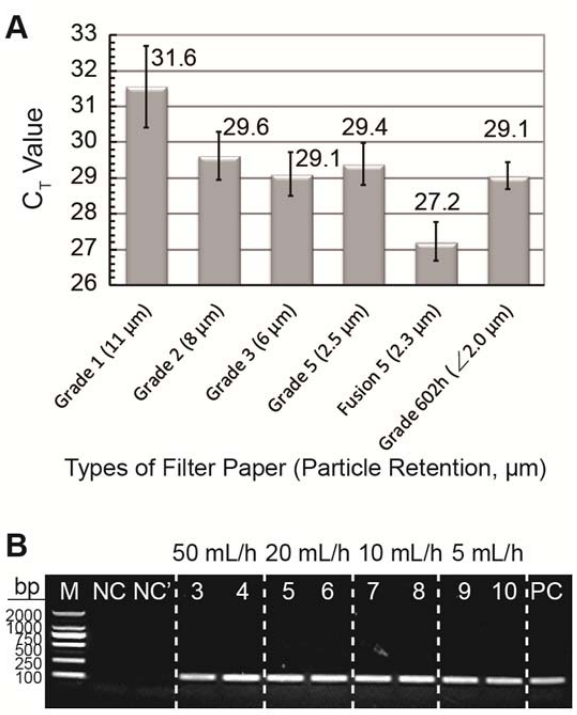


Figure 1.



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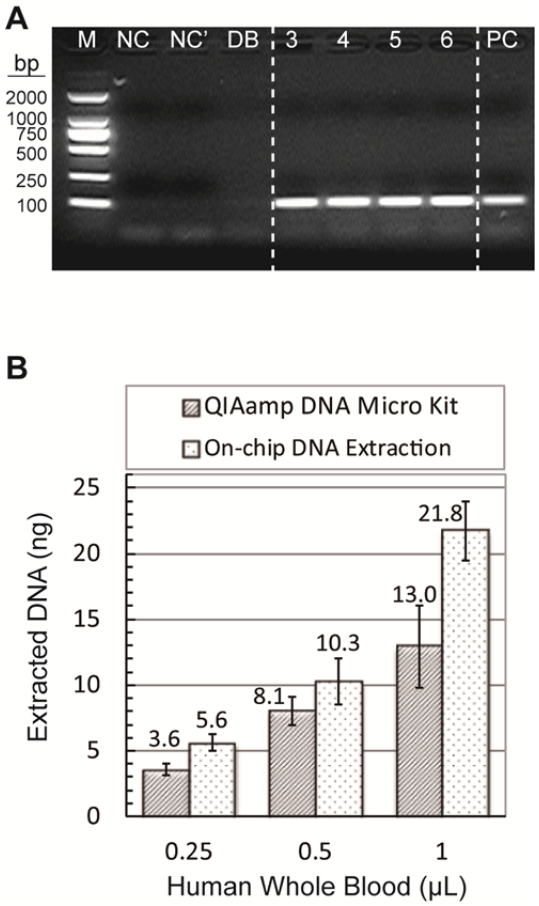

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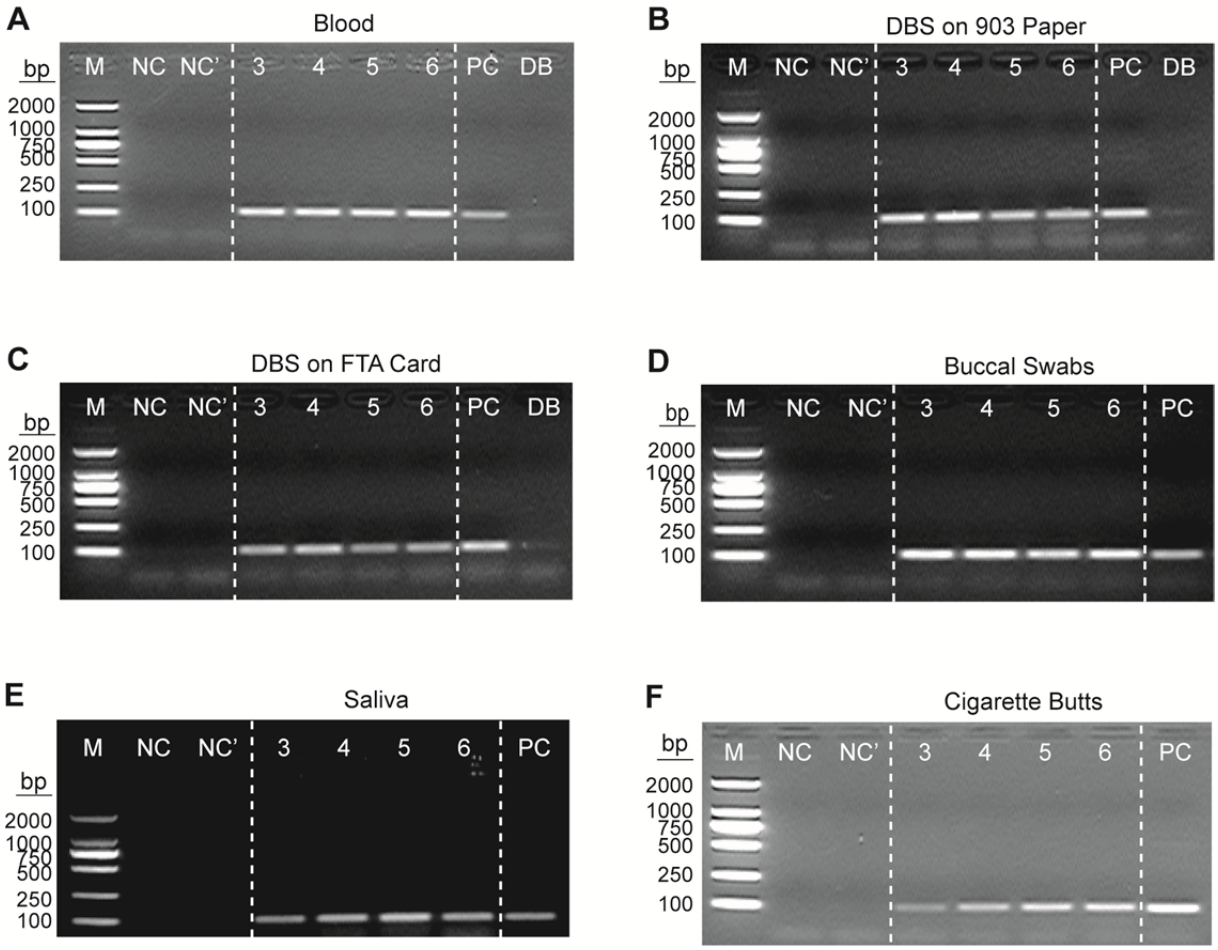

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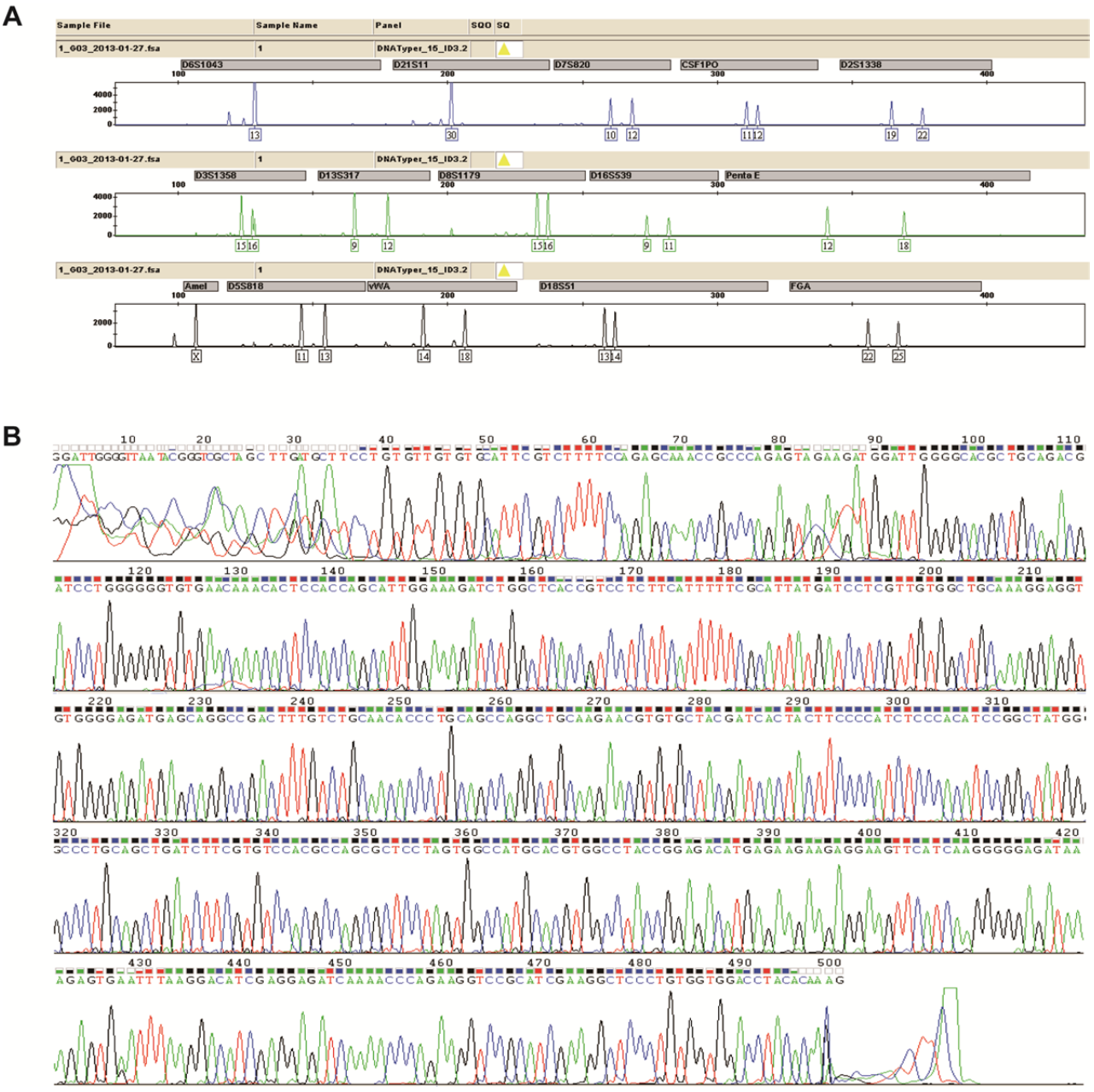

Figure 5.

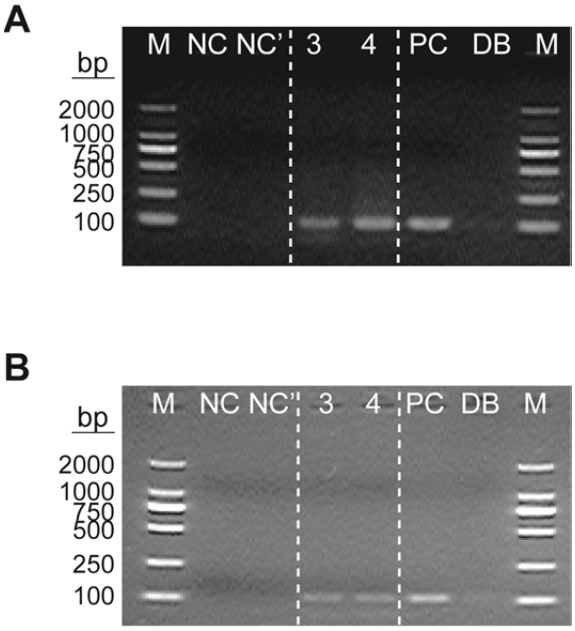

Figure 6.