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PAPER

A valveless microfluidic device for integrated solid phase extraction and polymerase chain reaction for short tandem repeat (STR) analysis

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A valveless microdevice has been developed for the integration of solid phase extraction (SPE) and polymerase chain reaction (PCR) on a single chip for the short tandem repeat (STR) analysis of DNA from a biological sample. The device consists of two domains—a SPE domain filled with silica beads as a solid phase and a PCR domain with an ~500 nL reaction chamber. DNA from buccal swabs was purified and amplified using the integrated device and a full STR profile (16 loci) resulted. The 16 loci Identifiler® multiplex amplification was performed using a non-contact infrared (IR)-mediated PCR system built in-house, after syringe-driven SPE, providing an ~80-fold and 2.2-fold reduction in sample and reagent volumes consumed, respectively, as well as an ~5-fold reduction in the overall analysis time in comparison to conventional analysis. Results indicate that the SPE-PCR system can be used for many applications requiring genetic analysis, and the future addition of microchip electrophoresis (ME) to the system would allow for the complete processing of biological samples for forensic STR analysis on a single microdevice.

Introduction

Most information gained from genetic analysis in the field of forensics derives from the interrogation of short tandem repeat (STR) regions in the genome. STRs are a highly discriminatory means for human identification because the number of repeats at a given loci can vary greatly between individuals.[1] Although STR profiles obtained from a DNA sample are a proven method for human identification, they are time consuming to obtain using conventional analysis methods. The processing time for a single sample requires 6–10 hours, contributing to low sample throughput in forensic laboratories which adds to the current national backlog, ~100 000 unanalyzed cases, that labs are facing.[2] A method for more rapid STR analysis would be beneficial to help increase sample throughput, therefore, decreasing the backlog, and allow investigators to more rapidly obtain vital information from DNA analysis. Microfluidics could be applied to forensic STR analysis to provide this rapid method for human identification. Microdevices provide an ideal environment for forensic STR analysis because points of sample exposure to the environment are greatly decreased, which reduces contamination and/or the presence of DNases, as the sample is contained within a closed environment throughout the entire analysis. Additionally, microfluidics inherently requires smaller reagent volumes, therefore, decreasing the cost-per-analysis. These advantages make microfluidics an ideal platform for forensic STR analysis.

The development of a forensic genetic micro-total analysis system (μTAS[3]) requires the integration of multiple sample processing steps including SPE, PCR, separation and detection, all miniaturized on a single microdevice. Numerous single process microdevices have been developed with only select focus on integration of multiple processes. One example of a fully integrated microfluidic system for genetic analysis was developed by Easley *et al.*,[4] demonstrating sample-in, answer-out capability for single-plex amplification and detection of *B. anthracis* and *B. pertussis*. Much other work toward development of an integrated microfluidic system for STR analysis has pertained to the separation and detection of PCR products[5–10] and integration of PCR with ME,[5,11] with little effort towards front-end DNA purification. The integration of SPE for DNA purification with downstream PCR was demonstrated by Bienvenue *et al.*,[12] but thermocycling was performed using a conventional thermocycler. This approach did not harness the potential for time-reduction inherent to microfluidics and corresponding technological advances in instrumentation.[12] As a result, it is important to further develop methods for integration of sample preparation (SPE) with the latter processes.

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Silica has been well-characterized as a reproducible, reliable solid phase for the purification of both DNA[4,13–19] and RNA[20] from biological sources in a microfluidic device for clinical and forensic purposes. Previous work has detailed the first example of integrated sample purification utilizing silica as a solid phase followed by amplification on a single microdevice for forensic analysis.[12,21] However, this work involved the use of a conventional thermocycler for PCR and relied on conventional thermal cycling times, therefore, not resulting in any significant decrease in analysis time. By incorporating a thermal cycling method involving non-contact, IR-mediated heating for performing PCR on a microdevice,[23–25] sample processing could be made significantly more rapid. IR-PCR utilizes a focused heating source and cooling fan to perform temperature cycling, allowing for fast temperature transitions. The faster heating and cooling rates provided by IR-mediated heating, in combination with the smaller volumes used in microfluidic devices compared to conventional PCR reaction volumes, provides a substantial decrease in the overall reaction time.[22,23]

The experiments detailed below describe the first integration of SPE and PCR on a single device, utilizing non-contact, IR-mediated PCR for a multiplex amplification using commercially available forensic STR amplification kits. Initial characterization of the SPE domain of two generations of an integrated device is shown, demonstrating the experimental timing necessary to trap the ideal elution fraction inside the PCR chamber of the device for amplification. The integration of SPE and PCR on a single device is first demonstrated for the analysis of miniSTRs from buccal swab samples using the commercially available MiniFiler™ amplification kit. MiniFiler™ uses primer sets that anneal closer to the repeat region (therefore, generating smaller amplicon sizes), and was developed specifically for very degraded samples. It is often used in tandem with other STR kits such as Identifiler™ to provide additional genetic information if a partial Identifiler™ profile results from analysis of a degraded sample. Optimization studies were performed to improve the device design, as well as to decrease the amplification time using faster DNA polymerases. Finally, SPE and IR-mediated PCR were performed on a single device, demonstrating the successful simultaneous amplification of sixteen loci [including the 13 core CODIS (Combined DNA Index System) loci] using the Identifiler® STR amplification kit in ~50 min. With further optimization and inclusion of microchip electrophoresis for separation and detection of the PCR products, a complete microfluidic system for STR analysis will soon be a reality, providing a fully integrated portable genetic analysis system for the forensic community.

Materials and methods

Reagents

Hyperprep silica beads (15–30 μm) were purchased from Supelco (Bellefonte, PA). Silica beads (5–15 μm) were purchased from Fuji Silysia Chemical Ltd (Aichi, Japan). Guanidine hydrochloride (GuHCl), 2-amino-2-(hydroxymethyl)propane-1,3-diol–HCl (Tris–HCl), isopropanol (IPA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 2-(4-morpholino)-ethane sulfonic acid (MES), Takara SpeedSTAR™ HS DNA Polymerase and Fermentas PyroStart™ 2X Fast PCR Master Mix were purchased from Fisher (Fairlawn, NJ). SigmaCote® was purchased from Sigma-Aldrich (St Louis, MO). Potassium chloride was purchased from Malinckroft (Paris, KY). Quant-iT™ PicoGreen® DNA reagent was purchased from Invitrogen (Carlsbad, CA). AmpF/STR® MiniFiler™ and AmpF/STR® Identifiler® amplification kits were purchased from Applied Biosystems (Foster City, CA). Human genomic DNA was purified from whole blood (University of Virginia Medical School) in-house. All solutions were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA). Buccal swabs were obtained from anonymous donors through a University IRB-approved protocol.

Microchip preparation

Using standard photolithographic techniques, PCR and integrated SPE-PCR devices were fabricated with Borofloat glass (Telic Company, Valencia, CA).[24] The resulting channel dimensions of the first generation integrated SPE-PCR device (Fig. 1) were SPE: 1 cm effective length, 200 μm deep with a line width of 150 μm; side arm: 200 μm deep, waste arm: 50 μm deep, and a weir (more shallow, etched region) 5–20 μm deep to allow for packing of the solid phase in the SPE channel; PCR: 200 μm deep with an ~500 nL chamber volume. An additional chamber (reference chamber) was etched with the same dimensions parallel to the PCR chamber to allow for temperature

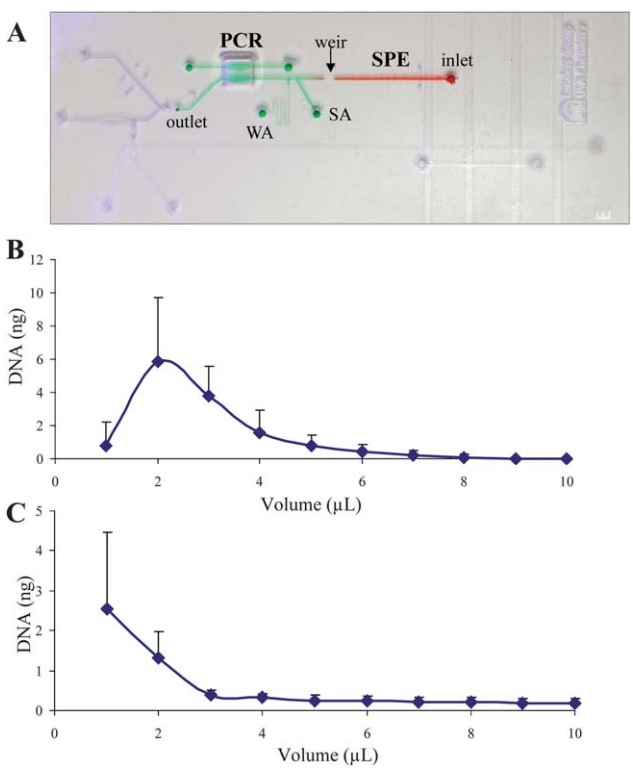

Fig. 1 First generation SPE-PCR device design. (A) SPE-PCR device design. (B) Elution profile of DNA from a buccal swab sample on SPE-PCR device using 15 μm silica. (C) Elution profile of DNA from a buccal swab sample on SPE-PCR device using 30 μm silica. Both bead sizes provided similar recoveries of DNA.

monitoring during infrared (IR)-mediated heating. A 1.1 mm diameter diamond-tip drill bit (Crystallite Corp., Lewis Center, OH) was used to drill access holes at the SPE inlet, side arm (SA), and waste arm (WA) while a 0.7 mm diameter diamond-tip drill bit was used to drill access holes for the outlet. A Borofloat glass cover plate was cut to fit the device and thermally bonded to the etched bottom plate. A window, 100 μm deep, was etched around the PCR chamber to reduce thermal mass after bonding each device to a glass cover plate. After fabrication, and prior to each amplification, the device was dried and passivated with Sigma-Cote®. For SPE, 30 μm silica beads were suspended in water and packed against the weir in the channel using vacuum. When 15 μm beads were used, a frit of 30 μm beads ~1 mm in length was first packed against the weir before filling the remaining portion of the channel with 5–15 μm silica particles. The channel was filled with new silica beads prior to each extraction to eliminate carry-over between samples.

A separate PCR (containing only the PCR domain) device, for master mix optimization studies, was fabricated with dimensions the same as those mentioned above for the PCR chamber in the integrated SPE-PCR device with a 200 μm depth and a 500 nL chamber volume. A reference chamber was also etched parallel to the PCR chamber for temperature monitoring. Following thermal bonding and etching of the window, access holes, 1.1 mm in diameter, were drilled at both ends of the channels. After fabrication, and prior to each amplification, the device was dried and passivated with SigmaCote®.

The second generation integrated SPE-PCR device (Fig. 3A) channel dimensions were 225 μm deep with a bottom width of 200 μm for both the SPE and side-arm channels. The SPE channel and side-arm channel had an effective length of 1.5 cm and 1.8 cm, respectively. The channels surrounding the PCR chamber were 200 μm deep, with a bottom width of 75 μm and 1.5 cm in length on each side of the 500 nL PCR chamber. A 500 nL reference chamber was etched with a channel of 4 mm for thermocouple insertion on the sample outlet end of the device, and a 5 mm channel on the opposite side. A window 100 μm deep was etched around the PCR and reference chambers to reduce thermal mass after bonding each device to a glass cover plate. Access holes, 1.1 mm in diameter, were drilled at the ends of all the channels. After fabrication, and prior to each amplification, the device was dried and the surface passivated with Sigma-Cote®. The SPE channel was packed with new 30 μm silica beads prior to each extraction to eliminate carry-over between samples.

Apparatus

The microchip-solid phase extraction (SPE) apparatus consisted of a SP100i syringe pump (WPI, Sarasota, FL) with a 250 μL Hamilton gastight syringe (Hamilton, Las Vegas, NV). PEEK™ tubing and mini-tight fittings (Upchurch Scientific, Oak Harbor, WA) were then used for connection of the syringe to the microchip.

The IR-PCR system consisted of an in-house built apparatus as previously described.[25] The system utilizes a halogen lamp to heat and a fan to cool, all controlled by LabVIEW programming to run a thermal cycling program. A thermocouple was inserted into a reference chamber containing 1× PCR buffer alongside the sample chamber to monitor and control temperature in the device. Mineral oil was then placed over the reference chamber and sample outlet reservoirs to prevent solution evaporation.

Microchip-solid phase extraction procedure

The solid phase extraction domain of the first generation integrated SPE-PCR microdevice filled with silica beads was conditioned with 6 M GuHCl, pH 6.1 for 10 minutes prior to each extraction. All steps in the extraction procedure with the first generation SPE-PCR device were performed at a flow rate of 5.23 μL/min, except for the elution step which was performed at 2.5 μL/min. Buccal cells were eluted from a cotton swab by vigorously mixing in 1 mL of 6 M GuHCl, pH 6.1 for 1 minute. This solution was vortexed for 1 min, and 400 μL aliquotted and diluted up to 1 mL with 6 M GuHCl, pH 6.1. The resulting solution was vortexed for 15 s and ~30 μL of this solution containing buccal cells was loaded onto the SPE domain. A 52 μL wash of 80% IPA (80/20 (v/v) IPA/water) was flowed over the silica bed to remove PCR inhibitors, cellular debris, and protein. The elution procedure consisted of the following steps: 2× PCR buffer was flowed in from the side arm (2.5 μL/min) and through the PCR chamber for 1 min 10 s. The side arm flow was stopped, and 2× PCR buffer was flowed through the SPE inlet (2.5 μL/min) until 1 μL emerged from the waste arm [the PCR chamber outlet was simply closed off using PDMS (polydimethylsiloxane) press-sealed over the outlet]. At this point, the side arm syringe was again started (the PDMS was removed to allow flow through the outlet) and twenty 1 μL elution fractions were collected for fluorescence analysis. For integrated SPE-PCR analysis, water was flowed through the side arm during the load and wash at 5.23 μL/min with all flow exiting through the outlet. The waste arm and PCR chamber were rinsed with the PCR master mix to eliminate any PCR-inhibiting compounds prior to elution. The MiniFiler™ reaction mix was then flowed until 1 μL exited the outlet and the side arm was then flowed (containing PCR master mix) in addition to the SPE inlet simultaneously (at a 1 : 1 ratio) until 1 μL exited the outlet (until the optimal fraction of the eluted DNA was located and held in the PCR chamber using PDMS press-sealed over the outlet).

With the second generation integrated SPE-PCR device, a slight variation of this procedure was performed. The flow rate of the condition, load, and wash step was increased to 7 μL/min due to the larger channel dimensions of this device, as well as the flow rate of water through the side arm during these steps. The silica bed was conditioned for 10 min with 6 M GuHCl, pH 6.1 prior to each extraction. Cells from a buccal swab were eluted in 980 μL of 6 M GuHCl, pH 6.1, and 20 μL proteinase K (20 mg/mL), incubated in a water bath at 56 °C for 10 min, and then 15.4 μL of the swab eluate was loaded onto the device. A 70 μL wash of 80% IPA was performed following the load. To elute the DNA, the flow rate of both the SPE and side arm was also reduced to 2.5 μL/min (as performed on the first generation SPE-PCR device). The MiniFiler™ or Identifiler® reaction mix was flowed through the SPE bed to elute the DNA at a 1 : 1 ratio with water simultaneously flowing through the side arm while 1 μL fractions were collected at the outlet for fluorescence analysis. For integrated SPE-PCR analysis using this second generation device, all the steps of the procedure were kept the same as described above, except PCR master mix was flowed through the

side arm during the elution. In order to trap the optimal fraction within the PCR chamber during the elution, both the SPE and SA were flowed until 4.5–4.75 μL had been collected from the outlet, and both flows were then stopped.

Fluorescence detection and sample amplification

Fluorescence detection was performed on a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE) using a Pico-Green® fluorescence assay. Sample amplification was performed on an IR-PCR system built in-house using the following protocols. PCR was initially performed using an in-house developed master mix containing MiniFiler™ primers and MiniFiler™ reaction mix in a 1 : 1 (v : v) ratio, and 0.12 U/μL AmpliTaq Gold® DNA polymerase. For PCR tests, this mixture was mixed 1 : 1 (v : v) with template DNA, and for integration was flowed in a 1 : 1 ratio with the eluting DNA. Cycling conditions consisted of: 94 °C for 660 s (initial denaturation), 32 cycles of denaturing at 94 °C for 5 s/annealing at 59 °C for 120 s/extension at 72 °C for 60 s, followed by 72 °C for 600–1200 s (final extension). When the amplification was reduced to 1.5 hours, the AmpliTaq Gold® was increased to 0.36 U/μL in the master mix, and the annealing and extension were reduced to 10 s each. The final extension was also reduced down to 1 min. For rapid Identifiler® amplification, the master mix recipe consisted of 1X PyroStart™ Fast PCR Mix, 2 μL Identifiler® primers, 0.125 U/μL SpeedSTAR™ HS DNA Polymerase, and 0.5 mg/mL BSA combined in a 1 : 1 ratio with template DNA. Cycling conditions: 94 °C for 60 s (initial denaturation), 32 cycles of denaturing at 94 °C for 5 s/annealing at 59 °C for 10 s/extension at 72 °C for 20 s, followed by 72 °C for 60 s (final extension). PCR products from all amplifications were separated and analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Results and discussion

As discussed above, previous work has been completed towards the integration of SPE-PCR on a single microdevice for genetic analysis.[12,21] Initial progress showed application of a fully integrated device for detection of *Bacillus anthracis* or amplification of a portion of the gelsolin gene. Although this work also employed IR-PCR for rapid thermal cycling, only single-plex amplifications were completed.[21] A next-generation SPE-PCR device was then developed for forensic STR analysis using commercial amplification kits, COfiler® and Profiler®. Although this showed application to multiplex amplifications, the numbers of loci amplified were only 7 and 10.[12] This required that both amplifications be completed in order to analyze all 13 core CODIS loci. Additionally, a conventional thermocycler was used for heating and cooling, resulting in slower temperature transitions and, therefore, longer amplification times. The research described herein aims to further the advancement of microfluidic technology for integrated analysis by concentrating on development of an improved SPE-PCR method capable of amplifying a greater number of loci in a shorter period of time. This is demonstrated successfully with two commonly used STR amplification kits, including MiniFiler™, for degraded sample types, and Identifiler™, which amplifies 16 loci simultaneously (including all 13 core CODIS loci) for greater discriminatory power.

First generation device design and elution profile studies

In order to achieve optimal amplification of all STRs, a sufficient quantity of template DNA must be present in the PCR reaction. In addition, while these devices will eventually be of single-use and disposable when utilized for forensic applications, for research purposes, the devices were cleaned and reused. Consequently, a solid phase first needs to be selected for the SPE domain of the integrated device that would provide the mass of DNA necessary for downstream PCR while also allowing for simple device preparation and easy removal from the device after each extraction. Both 15 and 30 μm silica beads were tested as a solid phase in the first generation SPE-PCR device as described in *Microchip-solid phase extraction procedure* in *Materials and methods*. DNA was extracted using each phase ($n = 3$ each) on the device from buccal cells eluted from a cotton swab and fluorescently analyzed to determine the DNA content in the elution fractions collected. It can be seen in the elution profiles in Fig. 1 that although 15 μm beads should have a higher capacity for DNA than 30 μm beads based upon the ratio of surface area to volume, both the 15 (Fig. 1B) and 30 (Fig. 1C) μm silica result in a statistically similar (±one standard deviation) recovery of DNA [13.6 (±7.5) ng and 5.9 (±2.3) ng, respectively] in the elution. This can be likely attributed to the protein from the buccal swab lysate binding to the phases during the load step (which has been previously shown to occur by Tian *et al.*[14]), blocking binding sites on the silica and, therefore, hindering the binding of DNA. Although the 15 μm silica phase has a higher surface area to volume ratio and, therefore, a higher capacity for binding DNA, the mass of protein present in the sample binding to the phase does not allow for a higher mass of DNA to bind to this phase compared to the 30 μm silica phase.

Based upon these results and because the 30 μm silica was easier to unpack from the devices than 15 μm silica, the 30 μm phase was chosen for all future studies. Additionally, the use of microchip SPE and 30 μm silica provided a concentration enhancement of 3.75-fold relative to the concentration of DNA in the starting buccal swab lysate. The elution profiles resulting from investigations into which phase would be optimal for further studies not only solidified the selection of 30 μm silica as the optimal phase but also indicated the optimal elution fraction for capture within the PCR chamber during the integration of SPE-PCR using this chip design. Fig. 1C depicts that the elution fraction that contained the largest quantity of DNA is fraction 1, or the first microlitre of the elution. However, residual IPA (a PCR inhibitor) from the wash step is pushed out from the SPE bed during elution, and is typically present in the first elution fraction (as shown by Easley *et al.*[4]) inhibiting PCR, so fraction 2 was chosen as the ideal fraction for amplification. To achieve a 1 : 1 ratio of DNA to master mix (which was determined to be optimal in tube studies—data not shown), both the SPE elution buffer and PCR master mix must be flowed at the same volumetric flow rate. The optimal timing of the elution and mixing of PCR master mix must also result in fraction 2 of the eluted DNA in the chamber (leaving ~250 pg DNA in the chamber). To achieve this timing during integration of SPE and PCR on this

device, a total of 1 μL eluate should be collected from the device outlet.

Integration of SPE-PCR

Microfluidic devices provide the optimal setting for forensic genetic analysis as points of contamination are reduced due to the closed environment in the microdevice throughout purification and amplification. Further, the development of an integrated microfluidic device capable of DNA purification and PCR amplification can provide the next advancement in genetic analysis for the forensic community as the chemistry that can be achieved in the small volumes inherent to microfluidic devices would allow for both processes to be carried out in a shorter time period than conventional analysis, therefore, increasing the sample throughput of forensic laboratories. Microfluidic devices also consume smaller reagent volumes than conventional analysis which decreases the cost-per-analysis. Most importantly, because of the small footprint of microfluidics they are ideal for being developed into portable technology which further decreases the time required for analysis.

Following determination of the optimal solid phase for use within the integrated device, the integration of SPE with non-contact IR-mediated PCR was performed on the first generation SPE-PCR device with a MiniFiler™ amplification. SPE was performed using 30 μm silica as described in *Materials and methods*, for the extraction of DNA from the buccal swab eluate. Upon trapping fraction 2 in the PCR chamber, along with the appropriate master mix, MiniFiler™ amplification was performed as described in *Materials and methods*. Separation and detection of resulting PCR products was performed on an ABI 310 Genetic Analyzer. The successful integration of DNA extraction and amplification on a single microdevice can be seen in the STR profile in Fig. 2. A total of 7 core loci and the sex marker amelogenin were amplified—with the dropout of one locus, D16. Although not a complete MiniFiler™ profile, with some adenylation issues and extraneous peaks observed (not a result of contamination and reduced in later master mix development), these results still demonstrate the first on-chip integration of SPE-PCR for the amplification of miniSTRs using non-contact IR-mediated heating. The STR profiles obtained from the analysis of multiple buccal swab samples ($n = 3$, data not shown) demonstrated the reproducibility of the system, with 8 loci effectively amplified but with dropout of locus D16. This type of allelic dropout has been reported in the literature when a decreased mass of DNA template is provided for amplification.[26] This is the case for the results shown in Fig. 2, where the greatest mass of DNA available for amplification in the PCR chamber was ~250 pg, which is at the lower limit of template considered optimal for MiniFiler™ by the manufacturer. A mass of 250 pg DNA in the chamber is also only achieved if the appropriate elution fraction (a volume ~500 nL) is trapped in the chamber. The combination of lower template mass, literature precedent for allelic dropout, and difficulty in integration of these processes on a microdevice without accurate flow control contributes to the dropout of D16 during this amplification.

Although allelic dropout was observed, the PCR amplification was reduced by ~30% [from 3.5 hours (conventional) to 2.5 hours (chip)], by utilizing the faster temperature transitions of the IR-PCR system as well as by reducing the denature time from 60 s to 5 s and the final extension from 45 min to 20 min. The 1.6-fold reduction in total analysis time here (for both SPE and PCR) from that required for conventional methods provides a clear advantage over conventional sample purification and

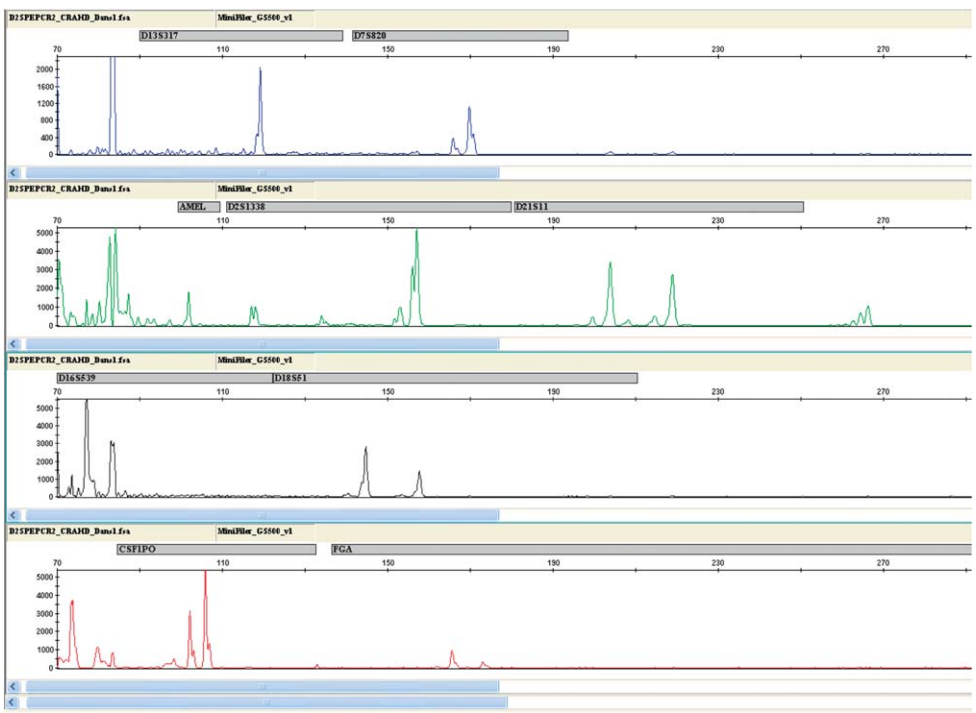

Fig. 2 MiniFiler™ STR profile resulting from a 1 : 1 ratio mixture of buccal swab DNA template to PCR master mix after integration of SPE-PCR utilizing IR-PCR on the first generation SPE-PCR device. Eight of nine MiniFiler™ loci are present after a 2.5 hour amplification.

amplification systems (Table 1). The SPE-PCR device developed also uses 40-fold less sample volume than conventional SPE and PCR analyses, making it advantageous in forensic cases where sample may be limited. The chip-based SPE provided a 3.75-fold concentration of the DNA, which is also important for forensic DNA analysis where template in the starting sample is dilute. Also, the method uses substantially less volume of reagents (1.4-fold) than conventional analysis, making the device more cost-effective for forensic DNA analysis.

Development and optimization of a second generation SPE-PCR microdevice

One approach for eliminating the allelic dropout associated with the SPE-PCR integration of MiniFiler™ where 8 loci were present (7 core and amelogenin) of 9 (Fig. 2) was to increase the mass of DNA available for amplification. This was investigated because, as previously mentioned, only ~250 pg would be present in the PCR chamber which represents a lower limit defined by the manufacturer.[27] To test this, the integrated chip was redesigned to incorporate larger channel dimensions for the SPE domain (Fig. 3A). A larger channel can hold a greater mass of 30 μm silica beads, allowing for an increase in the DNA binding capacity and an increase in the mass of DNA bound and recovered, therefore, increasing the template in the PCR chamber closer to 0.5 ng (mid-range of that recommended by the manufacturer, 0.25–0.75 ng[27]). Additionally, previous work has shown that, with an increase in channel dimensions (an increase in the quantity of the phase packed in the device), the DNA elutes in a larger volume and in a broader peak.[15,28,29] This would be beneficial, as it increases the window and volume range, containing concentrated DNA that can be trapped within the PCR chamber for amplification. Larger channel dimensions, and, therefore, a larger cross-sectional area, also allow for a faster volumetric flow rate to be used (still maintaining the same linear flow rate optimized for silica-based extractions[18]), decreasing the total analysis time. Elution studies were first performed to ensure the increased DNA capacity achieved through larger channel dimensions would also increase the mass of DNA recovered. DNA was purified from buccal cells using the second generation SPE-PCR device, and a fluorescence assay performed to determine the mass of DNA in each elution fraction. In the elution profile that resulted [Fig. 3B ($n = 3$)], it can be seen that an increased DNA recovery [15.9 (±5.1) ng] is indeed achieved through the use of the second generation device. The majority of the DNA elutes in the 5–6th μL (containing ~5 ng), which makes the timing of trapping the maximum amount of DNA in the PCR chamber easier, as there is a 2 μL window which contains ~4 to 5 ng/μL. This provides a much larger window of timing than with the previous generation device which had a 1 μL window that only contained ~2.5 ng. The shape of the elution profile from the second generation device indicates that a sufficient mass of DNA (0.25–0.75 ng) is available if DNA associated with the 5th–7th μL of the elution profile shown in Fig. 3B was trapped in the PCR chamber [providing 0.5–1.25 ng of DNA within the PCR chamber (well within the recommended range)]. This is again an advantage to using this chip design over the first generation SPE-PCR device, where the majority of the DNA eluted in the first microlitre, limiting the number of opportunities to trap a sufficient amount of DNA for amplification.

Table 1 Time and sample and reagent volume reductions provided by analysis using the SPE-PCR method in comparison to conventional analysis (*i.e.* Qiagen and conventional thermocyclers)

	SPE-PCR *vs.* conventional
Sample volume	40-fold reduction
Reagent volume	1.4-fold reduction
Amplification time	1.4-fold reduction
Total analysis time	1.6-fold reduction

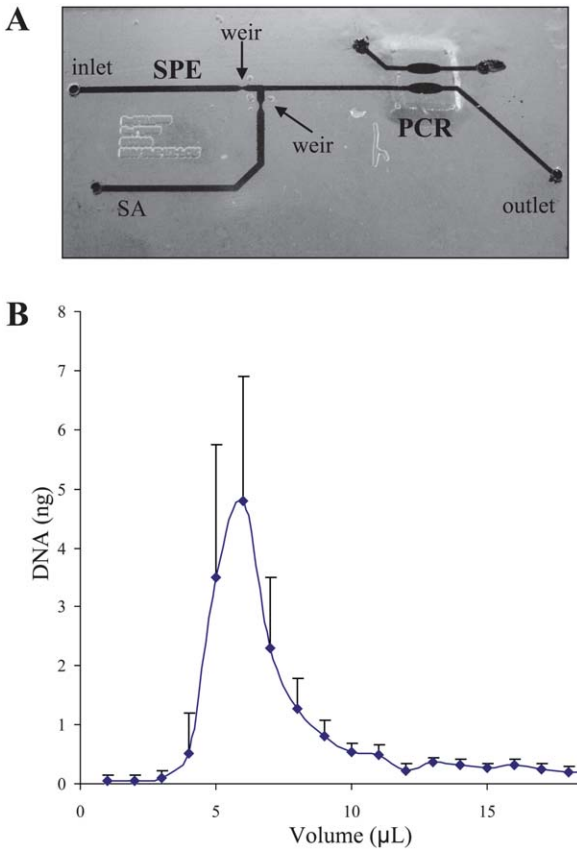

Fig. 3 Second generation SPE-PCR device design. (A) SPE-PCR device design. (B) Elution profile of DNA from a buccal swab sample on the SPE-PCR device using 30 μm silica.

Microchip PCR optimization for faster STR analysis

With a redesign of the integrated microdevice allowing for the input of more DNA into the PCR chamber, it was thought that the allelic dropout previously observed would no longer be a problem. In addition to increasing the mass of DNA for PCR to reduce the allelic dropout, approaches to further decrease the overall analysis time were explored. A major hindrance to the timely processing of DNA evidence is the lengthy analysis times for each step. By performing SPE on a microdevice, the time for DNA purification is reduced 4.5-fold (from 60 min down to <15 min). The lengthiest process though is the PCR amplification, which conventionally requires 3.5 hours. An overall decrease of 1 hour in PCR time alone was seen by moving to microchip-based PCR, harnessing the advantage of faster temperature transitioning inherent to the

use of the IR-PCR system utilized. To further reduce the time required for the MiniFiler™ amplification, each hold time in the thermal cycling protocol was further reduced. The initial denaturation of 11 min was kept consistent to ensure the proper length of time for heat activation of the AmpliTaq Gold® DNA polymerase used in this amplification. The denature, anneal, and extension steps were reduced to 5 s, 10 s, and 10 s, respectively, from the conventional times of 60 s, 120 s, and 60 s. The final extension was also reduced from 45 min to 1 min. After performing a microchip PCR MiniFiler™ amplification using these reduced thermal cycling times, the resulting STR profile (representative of $n = 3$), shown in Fig. 4A, was complete with all 9 loci (8 core and amelogenin) present. The time required for this amplification was 1.5 hours, a decrease of 2 hours compared to the 3.5 hour conventional MiniFiler™ amplification. With the successful amplification demonstrated here resulting in a complete STR profile, the next logical step was to test the integration of SPE and PCR on the second generation SPE-PCR device, which provided a greater recovery of purified DNA, incorporating the faster amplification times demonstrated here.

The integration of purification of DNA from a buccal swab with PCR amplification on a single device was performed, as described in *Materials and methods*. The ~6th μL of the elution curve, containing the maximum mass of eluted DNA, was trapped within the PCR chamber after elution (~1.25 ng), and the MiniFiler™ amplification performed (PCR time: 1.5 hours). A representative STR profile (of $n = 3$) which resulted (Fig. 4B) contains 8 (7 core and amelogenin) of 9 MiniFiler™ loci, with D16 missing and peak morphology issues with CSF1PO. It is interesting to note that this is the same locus that was missing from the STR profile resulting from the integration of SPE-PCR using the first generation device. The dropout of this locus is not a new observation. This particular locus has previously been reported with one of the largest dropout rates of all the core loci, even when increased injection times are used for the separation and detection of the amplified products.[26] The proclivity of the D16 locus to drop out, both in our results and those described in the literature, demonstrates the difficulty often seen when multiple processes are integrated into a glass microchip environment. To determine whether these issues were characteristic of the system with STR analysis, another commercial forensic STR kit, Identifiler®, was investigated.

Microchip PCR optimization of amplification using the Identifiler® kit

Due to the incomplete STR profiles resulting after MiniFiler™ amplification during the integration of SPE-PCR, another kit

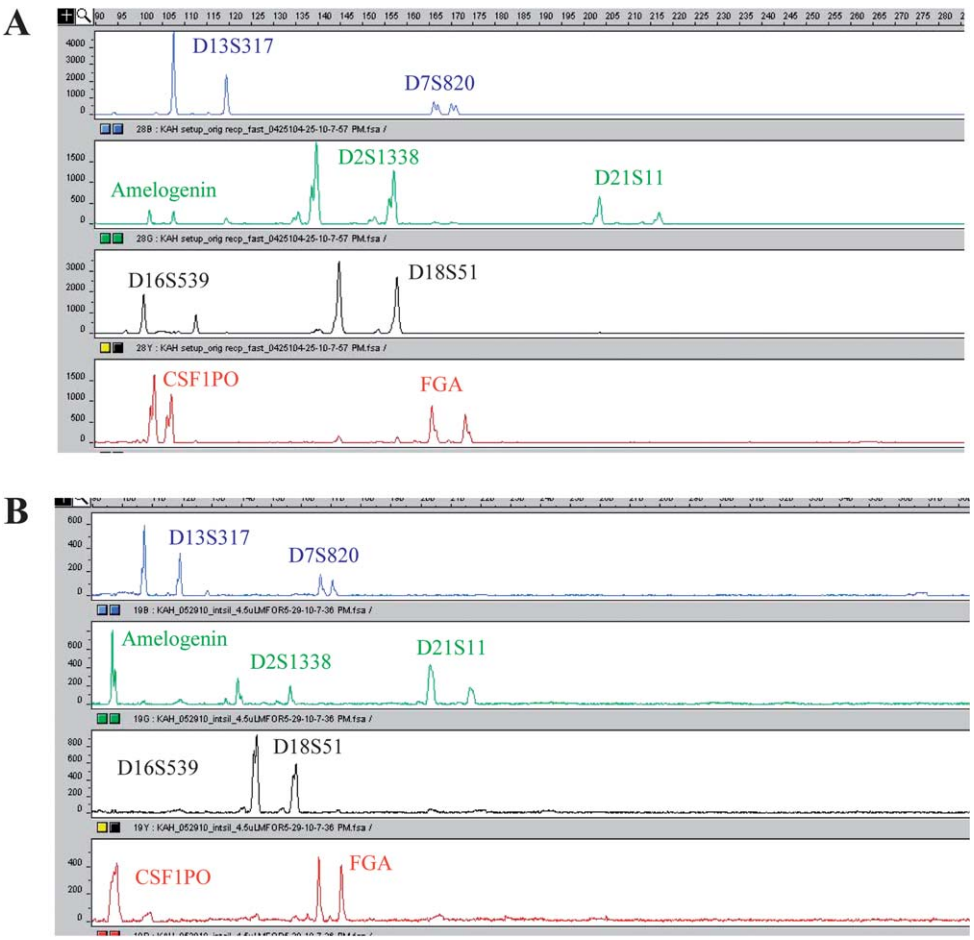

Fig. 4 (A) Complete MiniFiler™ STR profile from buccal swab DNA template performed in 1.5 hours on a microfluidic PCR device utilizing IR-PCR. (B) MiniFiler™ STR profile resulting after integration of SPE-PCR utilizing the second generation SPE-PCR device. Eight of nine MiniFiler™ loci are present.

commonly used for forensic human identification was investigated. First, a PCR master mix was developed incorporating the Identifiler® kit primer set with other reagents including a mixture of different DNA polymerases which would allow the amplification time to be further reduced. Identifiler® consists of primers for 16 loci, including amelogenin. Many forensic labs will use Identifiler® for initial DNA analysis, followed by MiniFiler™ to gain additional information on shorter fragments of loci that may amplify in MiniFiler™ but not with Identifiler®. The results from both kits are then combined to provide a higher power of discrimination for human identification.

Currently, the same DNA polymerase (AmpliTaq Gold®) is commonly used in all forensic amplification procedures even though faster polymerases are now available. This polymerase has an extension rate of 2.4 kb/min (the number of bases added to the growing DNA chain per minute held at the extension temperature) and 50–60 base processivity (the number of nucleotides that are added to the growing chain of DNA per association/disassociation of the polymerase with the DNA).[5] Faster enzymes on the market, such as PyroStart™ Fast PCR Master Mix (40 nuc/s) and SpeedSTAR™ HS DNA Polymerase (100 nuc/s), could provide up to a 3-fold increase in the speed of amplification over AmpliTaq Gold® (~33 nuc/s). In combination with the use of faster thermal cycling technology, this could allow for a great decrease in the amplification time and make DNA typing a more rapid process. To enhance the rapidity of the Identifiler® amplification, a new master mix recipe was developed using a mixture of these two fast polymerases (described in *Materials and methods*). The master mix for rapid amplification was based upon work completed by Vallone *et al.*,[30] which utilized both of the aforementioned fast polymerases. Some adjustments were made to the recipe for successful translation to the microdevice, as that completed by Vallone *et al.* was performed in a tube which has a smaller surface area to volume ratio than that of a microdevice. This can be problematic due to the increased potential of master mix components adhering to the channel walls. Because of this, the first component changed was the addition of BSA (bovine serum albumin) as it has shown by others to be beneficial for passivation.[31] The ratio of master mix components to solution containing DNA template was also adjusted from 1 : 3.4 to 1 : 1 so more DNA template would be trapped in the PCR chamber during integration.

Due to the faster extension rates of the two polymerases chosen, less time is needed at each step during thermal cycling. The initial denaturation was reduced from 11 min to 1 min, the denature, anneal, and extension reduced to 5 s, 10 s, and 20 s, respectively, and the final extension reduced to 1 min. Test amplifications ($n = 3$) were performed on a PCR microdevice utilizing the IR-PCR system, and in the resulting STR profile (Fig. 5), a complete STR profile, with 16 of 16 loci amplified and present, is shown which demonstrates the first 16-plex amplification on a microdevice. Although there are slight issues with adenylation and peak balance, it is still clear that a complete STR profile resulted. These issues involving profile quality, especially peak balance, will be addressed in future work with continued optimization of the multiplex amplification chemistry for microchip-based PCR. Thermal cycling was completed in 45 min, which is an ~5-fold decrease from the conventional amplification time. These results suggest promise that an Identifiler® analysis of a buccal swab, integrating SPE with PCR on a single device, could be completed in ~1 hour analytical time.

Identifiler® analysis of a buccal swab sample using integrated SPE-PCR

With a goal of performing rapid STR analysis using a microfluidic device for the integration of SPE-PCR, resulting in a complete STR profile, a buccal swab sample was processed using the second generation SPE-PCR microdevice. The new PCR master mix, incorporating faster polymerases with the Identifiler® primer set, was flowed through the side arm of the SPE-PCR device during the elution phase of SPE in a 1 : 1 ratio with the eluting DNA. Tests of different volumes of eluate collected from the outlet indicated that collecting 4.5 μL, effectively trapping the ~6^{th} microlitre in the PCR chamber, resulted in reproducible (16/16 loci callable above a threshold of 50 RFU) STR profiles. In Fig. 6, a representative STR profile from one of the integrations performed ($n = 3$) demonstrates the success of the SPE-PCR method. A complete Identifiler® profile is seen, with all 16 loci present and callable above a threshold of 50 RFU. This clearly demonstrates the effectiveness of the microfluidic method for the integration of SPE-PCR for STR analysis. When evaluating an STR profile, it is important to consider not only the presence of amplified alleles but also the quality of the amplification, including: whether signal intensity is above a set threshold for all expected loci, whether there is comparable signal intensity between loci (interlocus peak balance), heterozygote balance (intralocus peak balance), the absence of incomplete adenylation products, the absence of non-specific amplification products, and the absence of significant stutter artifacts. Further optimization of the method is necessary to achieve these characteristics, as the locus to locus balance especially could be improved. However, the method developed here is a workable foundation for future optimization studies and will lead to the development of a device for complete STR analysis, from DNA purification to microchip electrophoresis for PCR product separation and detection, which will make rapid STR analysis a reality for use in forensic laboratories. Although further optimization is necessary, the results presented show the first demonstration of integrated SPE-PCR using IR-mediated heating for the amplification of 16 forensic STR loci in ~1 h. This a significant decrease from the time required for conventional analysis, which would increase the sample throughput of forensic laboratories. The use of a microdevice also provides a completely closed environment from the time lysate is introduced to the completion of PCR amplification, which is ideal for avoiding sample contamination during forensic genetic analysis.

Conclusions and future work

The first integration of SPE and PCR on a single microdevice utilizing non-contact IR-mediated heating for an Identifiler® STR amplification that was detailed in this work provides an important step towards a fully integrated forensic genetic analysis system. Rapid STR analysis was demonstrated, highlighting the advantages inherent to microchips, and utilizing fast DNA polymerases to decrease the total analysis time to ~1 hour. The reproducibility achieved with this device from a highly variant

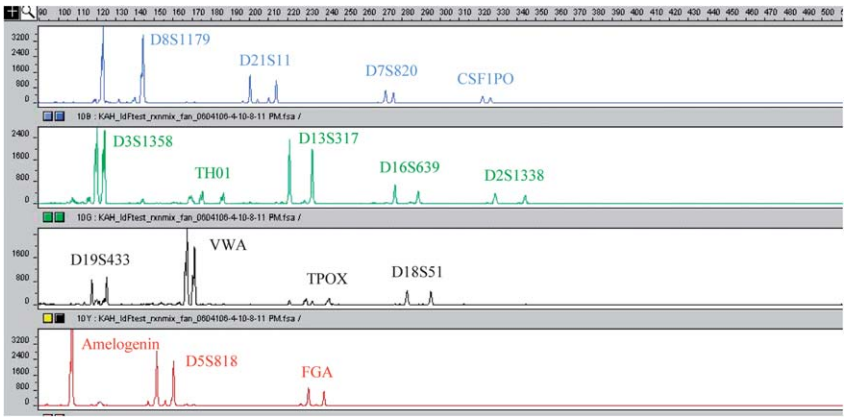

Fig. 5 Identifiler® STR profile resulting from a 1 : 1 ratio mixture of buccal swab DNA template to PCR master mix on a microfluidic PCR device utilizing IR-PCR. Rapid PCR was performed using a mixture of fast DNA polymerases, resulting in amplification of 16 Identifiler® loci in 50 min.

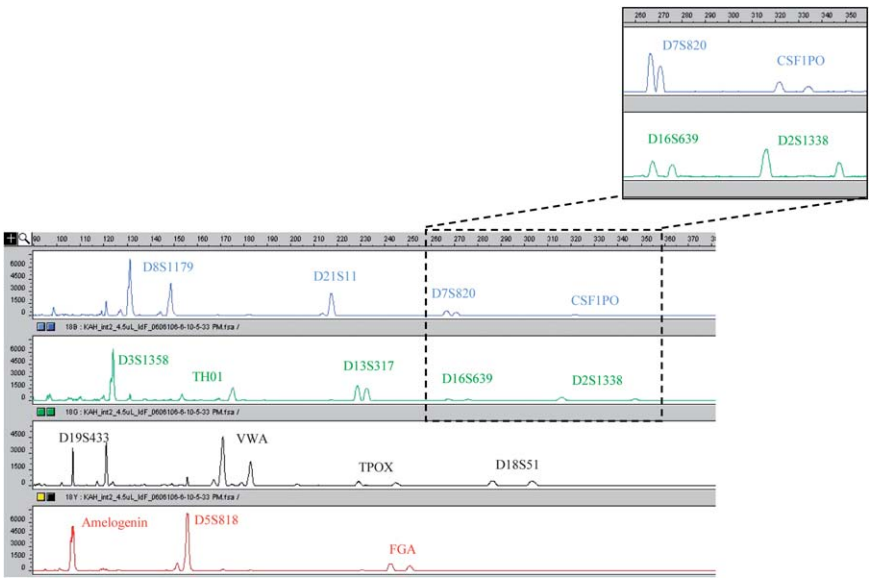

Fig. 6 Complete Identifiler® STR profile resulting from integration of SPE-PCR on the second generation SPE-PCR device. DNA was purified from a buccal swab and a rapid, 16 loci Identifiler® amplification performed in a total analysis time of ~1 hour.

sample type such as the buccal swabs used here demonstrates the potential for application to additional complex sample types. The robustness of the Identifiler® primer set was also realized when adjustments were made to the ratio of template DNA to master mix recommended for use for amplification by the manufacturer and a full STR profile was still obtained. Future work will involve further testing and optimization of the new SPE-PCR device design in addition to the integration of microchip electrophoresis into the device to provide the sample in–answer out capability of a microchip-STR genetic analysis system for the forensic community.

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