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Development of a real-world direct interface for integrated DNA extraction and amplification in a microfluidic device

Kirsty J. Shaw,[a] Domino A. Joyce,[b] Peter T. Docker,[a] Charlotte E. Dyer,[c] Gillian M. Greenway,[a] John Greenman[c] and Stephen J. Haswell*[a]

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Integrated DNA extraction and amplification have been carried out in a microfluidic device using electro-osmotic pumping (EOP) for fluidic control. All the necessary reagents for performing both DNA extraction and polymerase chain reaction (PCR) amplification were pre-loaded into the microfluidic device following encapsulation in agarose gel. Buccal cells were collected using OmniSwabs [Whatman™, UK] and manually added to a chaotropic binding/lysis solution pre-loaded into the microfluidic device. The released DNA was then adsorbed onto a silica monolith contained within the DNA extraction chamber and the microfluidic device sealed using polymer electrodes. The washing and elution steps for DNA extraction were carried out using EOP, resulting in transfer of the eluted DNA into the PCR chamber. Thermal cycling, achieved using a Peltier element, resulted in amplification of the Amelogenin locus as confirmed using conventional capillary gel electrophoresis. It was demonstrated that the PCR reagents could be stored in the microfluidic device for at least 8 weeks at 4 °C with no significant loss of activity. Such methodology lends itself to the production of 'ready-to-use' microfluidic devices containing all the necessary reagents for sample processing, with many obvious applications in forensics and clinical medicine.

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Introduction

Nucleic acid purification and amplification are important biochemical tools for genetic analysis. In many instances, such as forensic or clinical investigations, biological samples are limited both in terms of quantity and quality, accordingly retrieval of sufficient amounts of high enough quality DNA from the original sample without contamination is crucial. The use of solid-phase DNA extraction methodology is highly suited to such samples as it also enables pre-concentration of nucleic acids. Genomic regions of interest can then be amplified from this template DNA using PCR, facilitating simplified detection, for example through the incorporation of fluorescently labelled primers.

A wide variety of DNA purification protocols have been successfully adapted to work in a microfluidic environment. Silica-based, solid-phase methodology has been commonly employed in which nucleic acids adsorb onto the silica in the presence of a chaotropic salt. Cellular or proteinaceous debris can then be removed using an alcohol wash, and the nucleic acids subsequently eluted in a low ionic strength solution enabling direct transfer to downstream applications. In addition to silica-based methodologies, a range of other nucleic acid extraction techniques have been successfully applied to microfluidic systems, such as the use of organic polymeric monoliths and ion-exchange resins, each suited for different applications.[1]

PCR in microfluidic systems has been widely reported in the literature and the reader is directed for more information to substantial reviews which look in detail at the diverse variety of amplification systems which exist.[2–4] Whilst the integration of PCR with downstream analysis techniques (mainly capillary gel electrophoresis) has received significant interest, much less focus has been placed on the integration with pre-PCR procedures such as DNA extraction; important when dealing with crude biological samples.[5] This is in part because of the difficulties associated with confining the solid-phase extraction matrix within a specific location in the microfluidic device, preventing contamination of the template DNA with potential inhibitors from the DNA extraction process *e.g.* isopropanol, and isolating any surface coating required for the prevention of DNA polymerase adsorption onto the internal glass surfaces of the PCR chamber.[6]

A number of approaches designed to overcome the challenges associated with developing microfluidic devices for integrated DNA extraction and amplification have been evaluated. Silica-coated or surface-charge switchable magnetic particles can be used to facilitate nucleic acid movement within microfluidic systems. For example, Pipper *et al.*[7] used superparamagnetic particles to move viral RNA between aqueous droplets in a microfluidic environment. Each droplet, separated by an oil carrier phase, contained a different reagent solution for sequential nucleic acid purification and reverse transcription (RT)-PCR. Alternatively, the particles can be retained within one or more chambers on a microfluidic device using an external magnet, allowing the various biological sample, DNA extraction and PCR solutions to be pumped over the solid-phase surface.[8,9]

[a]*Department of Chemistry, University of Hull, Cottingham Road, Hull, UK HU6 7RX. E-mail: s.j.haswell@hull.ac.uk; Fax: +44 (0)1482 466410; Tel: +44 (0)1482 465475*
[b]*Department of Biological Sciences, University of Hull, Cottingham Road, Hull, UK HU6 7RX*
[c]*Centre for Biomedical Research, University of Hull, Cottingham Road, Hull, UK HU6 7RX*

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This enables nucleic acids present in the biological sample to bind to the particles during the extraction process, allowing them to be directly transferred into a chamber containing PCR reagents. However, it has been shown that performing PCR in the presence of magnetic beads can decrease the amplification efficiency by as much as 50%.[10]

Another approach is to combine the flow of DNA eluted from a solid-phase extraction matrix with a concentrated PCR reagent mixture from a side channel and direct the combined solutions into a PCR chamber for amplification.[11,12] Direct elution of RNA, from immobilised silica beads, using a nucleic acid sequence-based amplification (NASBA) reagent mixture has also been demonstrated, although in this instance the enzymes required for NASBA were added to the amplification chamber in a separate step following RNA elution.[13]

The use of mechanical external syringe pumps to facilitate flow of solutions through such integrated devices allows accurate control of flow rates but requires the use of miniaturised fittings to provide an interface to the microfluidic device, which in turn brings problems associated with large dead volumes (important when dealing with expensive reagents such as DNA polymerase) and labour intensive assembly.[14] The use of dynamic EOP, however, eliminates the need for moving parts enabling easier integration of pumping methodology into the microfluidic device. A recent review by Wang *et al.*[15] demonstrated the flexibility of electro-osmotic pumps for a variety of uses including microflow injection analysis and microfluidic chromatography systems. EOP has recently been demonstrated by the authors to be a suitable pumping technique for performing DNA extraction on a microfluidic device, using a dual function monolith which not only provides the solid-phase for DNA extraction but also acts as support for electro-osmotic flow (EOF) whilst reducing the reverse hydrodynamic flow of solutions.[16]

In addition to integrating pumping mechanisms onto microfluidic devices, the storage of reagents within such devices lends itself to the development of truly portable miniaturised systems. Long-term storage of reagents on microfluidic devices for performing nucleic acid-based reactions has received limited attention in the literature but in recent years the need for such capability has become apparent if truly portable, integrated microfluidic systems are to be developed. In terms of DNA extraction, storage of liquid reagents for washing and elution has been achieved by using valve actuated reservoirs[17] or glass ampoules.[18] The latter requires the application of a local mechanical force to break the ampoules and centrifugal force to ensure release of reagents to the desired location in the microfluidic device.[18] An alternative approach has been described by Yobas *et al.*,[19] who presented a self-contained cartridge which can be connected to a microfluidic device and contains all the necessary reagents for performing nucleic acid extraction. In this case a magnetic planar peristaltic pump was used to move reagent plugs, separated by air spaces, around the system. Whilst representing an advance in the development of 'ready-to-use' microfluidic technology, no long-term storage of the reagents was evaluated as the cartridges were used immediately following preparation. The use of a cartridge for reagents also represents an extra component in addition to the microfluidic device increasing the complexity of operation.

The storage of essential components for nucleic acid amplification within microfluidic devices has focussed on the drying of PCR reagents within amplification chambers. For example, by freeze–drying PCR reagents onto the internal surfaces of a PCR chamber, successful DNA amplification can be performed after six months storage at −20 °C.[20] However, when the PCR reagents were stored at higher temperatures, of 4 °C or room temperature, DNA polymerase was found to be half or completely inactive respectively. Furthermore, Kim *et al.*, have demonstrated that PCR reagents can be dried at room temperature and passivated using a protective paraffin layer which melts during thermal cycling facilitating release and hydration of the reagents.[21] More recently, the same group have combined this form of dry reagent storage with liquid storage of reagents for upstream DNA extraction.[22] Whilst the drying of PCR reagents within microfluidic devices eliminates the need for reagent loading at point-of-use, offering several advantages including reduced risk of contamination and increased portability, there are problems relating to decreased DNA polymerase activity as a result of the drying methodology utilised.[21]

The work presented here describes an integrated DNA extraction and amplification methodology in which all the required reagents are pre-loaded onto the microfluidic device by encapsulating them in agarose gel. A bi-functional thermally activated silica-based monolith was used as the solid-phase for DNA extraction and as a pump for electro-osmotic movement. The efficiency of the DNA extraction process was evaluated along with the effectiveness of encapsulation of PCR reagents in a gel-based format for storage on the microfluidic device.

Experimental

Microfluidic device

All glass microfluidic devices were produced using standard photolithography and wet etching techniques to generate the design shown in Fig. 1.[23]

The features were etched to a depth of 50 μm using a 1% hydrofluoric acid/5% ammonium fluoride solution at 65 °C for 10 minutes. In order to allow electrodes to be connected to the microfluidic device, 3 mm diameter holes were drilled in a 3 mm thick top plate which was then thermally bonded to the etched glass wafer (1 mm thick) to produce the complete microfluidic device. Injection moulded carbon-containing polystyrene plugs [N. Goddard, Manchester University, UK] were fitted into the holes and then brought into contact, *via* pogo-pins, with electrodes housed in the custom-made control instrument [JLS Designs Ltd, UK] shown in Fig. 2.

The internal glass surfaces of the PCR chamber were silanised to prevent DNA polymerase adsorption. A solution of 290 μl of trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane [Sigma-Aldrich, UK] in 5 ml of 2,2,4-trimethylpentane [Fisher Scientific, UK] was flowed through the microfluidic device for 10 minutes at 5 μl min^{-1}. Following this, solutions of 2,2,4-trimethylpentane, acetone and distilled water were sequentially used to wash the microfluidic device for a total of 5 minutes each at 5 μl min^{-1}.[24]

The channels of the DNA extraction chamber were incubated with a 5% hexadimethrine bromide (Polybrene, PB) [Sigma-Aldrich, UK] solution for 15 minutes and then dried under

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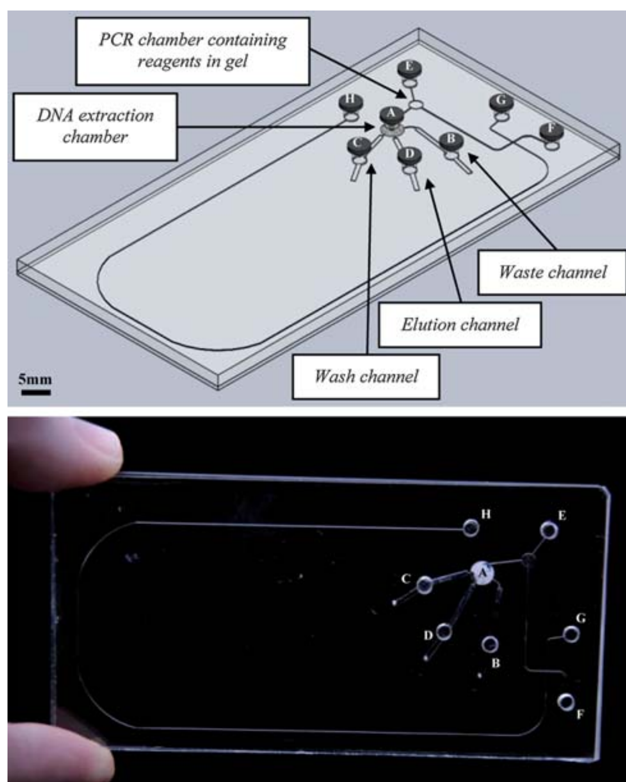

Fig. 1 Schematic (top) and photograph (bottom) of the microfluidic device showing the thermally activated silica monolith (A) within the microfluidic device, the position of the carbon electrodes (B–H) and the locations of the gel encapsulated reagents. The additional channel between electrodes G and H provides the potential for future integration with capillary electrophoresis for detection of PCR products.

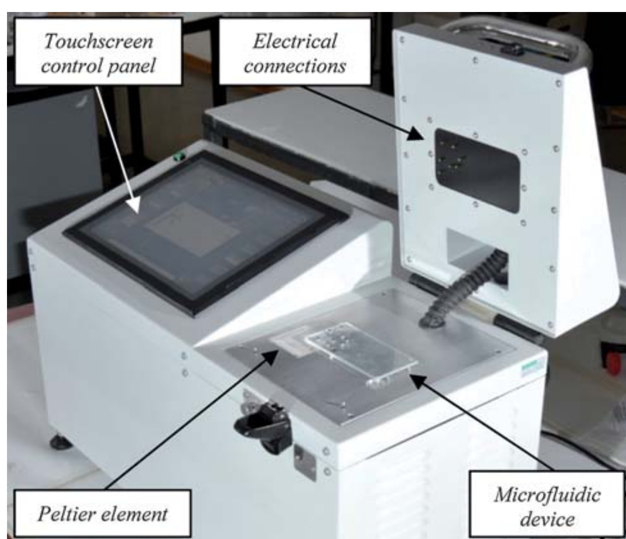

Fig. 2 Photograph of the control instrument, showing position of the microfluidic device, Peltier element, electrical connections and touchscreen control panel.

vacuum. This created a positively charged surface reversing the normal direction of EOP so that bulk movement occurs from cathode to anode.

The thermally activated silica-based monolith was produced by mixing potassium silicate solution (21% SiO_2 and 9% K_2O [VWR International, UK]) and formamide [Alfa Aesar, UK] in a 10 : 1 ratio.[25] In order to ensure the monolith was only produced in the DNA extraction chamber, the entire device was first filled with glycerol [Sigma-Aldrich, UK]. The monolith solution was then injected into the DNA extraction chamber, displacing the glycerol, and the microfluidic device placed in an oven at 90 °C for 15 minutes. After this initial heating step, the remaining glycerol was removed and the microfluidic device placed back in the oven overnight for complete polymerisation to occur. Once polymerised, the monoliths were washed with ethanol to remove any unreacted solution and glycerol residue. Prior to DNA extraction the monoliths were washed with 10 mM TE buffer (10 mM Tris and 1 mM EDTA in distilled water, adjusted to pH 6.7 using hydrochloric acid [Sigma-Aldrich, UK]) at 5 μl min^{-1} for 30 minutes.

All the necessary reagents for performing DNA extraction and amplification were encapsulated in 1.5% (w/v) low-melting temperature (LMT) agarose [Sigma-Aldrich, UK] for pre-loading and storage on the microfluidic device. The wash gel contained 50% (v/v) ethanol in 100 mM sodium chloride for increased electro-osmotic movement,[16] and the elution gel was made up using 10 mM TE buffer. For single locus amplification PCR reagents were added to a molten agarose solution (in which the concentration of the agarose gel was varied in the initial experiments[26]): 1× GoTaq® buffer, 2 mM $MgCl_2$, 1 unit GoTaq® Hot Start DNA polymerase [Promega, UK], 10 mg ml^{-1} bovine serum albumin [NEB Inc., UK], 0.01% (w/v) poly(vinylpyrrolidine), 0.1% (v/v) Tween-20 [Sigma-Aldrich, UK], 200 μM each deoxyribonucleotide triphosphate [Bioline, UK] and 0.1 μM Amelogenin forward and reverse primers[27] [Eurofins MWG Operon, Germany]. The molten wash, elution and PCR gel solutions were injected by positive pressure into the relevant locations on the microfluidic device through electrode loading holes C, D and E respectively, prior to electrode fitting (Fig. 1).

DNA extraction and amplification

Buccal swab DNA samples were collected using an OmniSwab™ [Whatman, UK] which was gently scraped along the inside of the cheek. The tip of the swab was then added to a binding/lysis solution of 5 M guanidine hydrochloride (GuHCl) [Sigma-Aldrich, UK] in 10 mM TE buffer previously added (20 μl) to the DNA extraction chamber (port A in Fig. 1). A carbon containing polystyrene plug was then placed into port A which enabled the DNA present in the binding/lysis solution to be displaced onto the surface of the silica monolith with the residual liquid passing into channel B shown in Fig. 1. An ethanol wash was used to remove any cellular or proteinaceous debris from the sample by applying a voltage of 100 V cm^{-1} between electrodes at B and C resulting in EOP of the ethanol wash gel contained in channel C (Fig. 1). The DNA retained on the monolith was then eluted into the chamber pre-loaded with PCR reagents by applying a voltage of 100 V cm^{-1} from electrodes D to E resulting in EOP of the elution solution contained in channel D (Fig. 1). During this step the PCR reagents were cooled to 4 °C using a thermoelectric Peltier element to prevent adverse thermal effects that may arise

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from Joule heating. DNA amplification was then performed using the same Peltier system, which provided both the heating and cooling required for thermal cycling, according to the following program: a Hot-Start step of 95 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds, with a 7 minute final extension at 60 °C. Control PCR samples were run on a Techne TC-312 thermal cycler, at the same temperatures and hold-times as the Peltier system.

DNA quantification

Quantification of the DNA obtained from the extraction procedure was performed using a Quant-iT™ PicoGreen® double stranded (ds) DNA Assay Kit [Invitrogen, UK]. To each 2 μl aliquot of sample from the DNA extraction process, 100 μl of the PicoGreen® working stock solution was added, based on manufacturer's protocol, in a black microtitre plate. DNA standards were used to provide a calibration curve at the following concentrations: 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 ng μl^{-1}. A blank containing no DNA was also used to account for any background fluorescence. All samples were analysed using a FLUOstar Optima Plate Reader [BMG Labtech, UK].

Analysis of PCR products

PCR products were analysed off-chip by capillary electrophoresis using an ABI Prism 310 Genetic Analyser [Applied Biosystems, UK]. The PCR-amplified DNA samples were collected from the DNA amplification chamber (Fig. 1) and added to 12 μl of Hi-Di™ formamide and 0.5 μl GeneScan™ 500 ROX DNA size standard [Applied Biosystems, UK]. The solutions were then heated to 95 °C for 5 min to denature the DNA before being snap-cooled and electrokinetically injected into the capillary.

Results and discussion

DNA recovery direct from buccal swabs

In order to reduce the complexity of sample introduction into the microfluidic device the direct transfer of DNA from the buccal swab to the extraction monolith was evaluated. This was carried out by placing the buccal swab tip (Fig. 3, section A) directly into port A, indicated in Fig. 1, which had been pre-loaded with 20 μl of binding/lysis solution. The buccal swab tip was left in the binding/lysis solution for varying amounts of time and the amount of DNA present in the lysate analysed. Optimum yields were produced when the swab tip remained in the binding/lysis solution for a total of 10 minutes (Fig. 4).

In order to ensure that the tip of the swab would yield sufficient amounts of DNA for analysis, the DNA yields for each segment of the swab were analysed. The average DNA concentration for each segment (A–G, as shown in Fig. 3) was found to be 0.576 ± 0.086 ng μl^{-1}, indicating that all sections of the buccal swab, including the tip, were equally effective in retaining DNA and could be used for analysis.

Previous work by the authors has shown that DNA extraction can be successfully performed using conventional EOP across a silica-based monolith.[28] Here DNA was eluted using an applied voltage of 100 V cm^{-1} resulting in DNA extraction efficiencies of

Fig. 3 Photograph showing the individual buccal swab segments that were analysed.

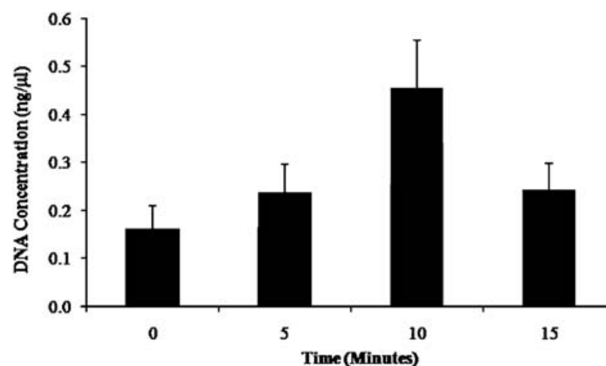

Fig. 4 Graph showing the effect of time on DNA recovery from buccal swab tips using a 5 M GuHCl lysis solution (error bars indicate standard deviation, $n = 6$).

approximately 52%. Despite sufficient DNA being recovered for PCR amplification, the DNA extraction efficiency was limited by the anionic nature of the DNA. As conventional EOF creates bulk movement in the direction of the cathode it opposes the natural electrophoretic migration of the DNA therefore reducing the overall mobility of the DNA. By reversing the direction of EOF, *i.e.* creating a positively charged surface, both EOF and electrophoresis work together to move DNA to the anode resulting in a more efficient process.

To this end, DNA extraction from monoliths using EOP in channels treated with PB was evaluated. The average DNA extraction efficiency obtained using an applied voltage of 100 V cm^{-1} was found to be 74.5% (with a standard deviation of 20.3%) representing an increased yield as compared with conventional forward EOP (data not shown).

Encapsulation of PCR reagents in LMT agarose gel

A range of final agarose gel concentrations, ranging from 0 to 2.5% (w/v), for encapsulating the PCR reagents were evaluated. Conventional aqueous PCR reagent solutions, containing no agarose gel, were used as control samples. All samples were amplified in a standard bench top thermal cycler and the relative fluorescence intensities of the PCR products, as determined by capillary electrophoresis, were compared.

Comparable PCR efficiencies were demonstrated when using agarose gel concentrations between 0 and 1.5%. However, at agarose gel concentrations of 2% or greater there was a significant decrease in the signal intensity observed, most likely due to high viscosity of the gel matrix slowing the diffusion rate of reactants during the primer annealing and DNA extension phases of the reaction.[26] Accordingly, a 1.5% agarose gel was identified as optimal for maintaining a high PCR efficiency, with

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the gel providing increased mechanical stability for reagent storage (Fig. 5).

Once the optimum agarose gel concentration had been established the stability of PCR reagents contained within the matrix was examined. All the necessary reagents for PCR, excluding the DNA, were incorporated into a 1.5% (w/v) agarose gel solution and stored at room temperature, 4 °C or −20 °C for up to 8 weeks. DNA was added immediately prior to analysis and the stability was determined by measuring the relative fluorescence intensity of the PCR products obtained (Fig. 6).

The results indicated that storage of the encapsulated PCR reagents at room temperature was not a viable option as the signal intensity is drastically reduced after just 24 hours. Reagents stored at either 4 °C or −20 °C, however, retain the ability to support PCR amplification of DNA at the same efficiency for at least 8 weeks. By storing the microfluidic device under these conditions, all the necessary reagents for performing PCR amplification of specific target loci can be pre-loaded onto the device.

Integration of DNA extraction and amplification

Following optimisation of the individual processes on the microfluidic device, integration of the two processes was evaluated for DNA extraction and amplification of a single target locus (Amelogenin). A small section of buccal swab was placed in port A directly above the silica monolith, along with 20 μl of binding/lysis solution, before being sealed using a polystyrene plug. The entire microfluidic device was then placed inside the control instrument. Following a 10 minute incubation step at room temperature, voltages were applied to achieve movement of the reagents stored on the microfluidic device for both washing of the monolith and subsequent DNA elution. Once the DNA had been transferred into the PCR chamber, which contained all the necessary reagents for amplification, thermal cycling was performed using the Peltier heating/cooling system. Successful amplification of the target locus directly from a buccal swab using the self-contained microfluidic device was confirmed using conventional capillary gel electrophoresis (Fig. 7).

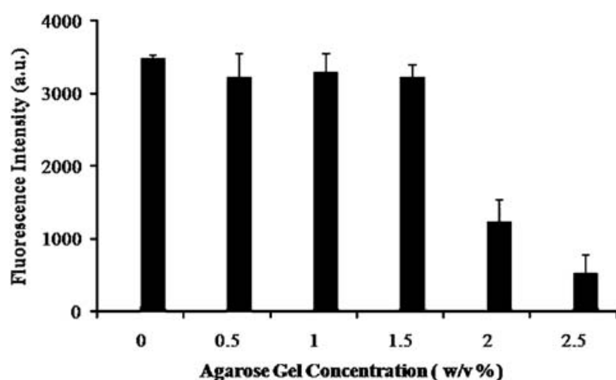

Fig. 5 Amplification of a single locus (Amelogenin) from male DNA with PCR reagents encapsulated in different concentrations of agarose gel. Samples were analysed using capillary electrophoresis and the peak heights of the two alleles (XY) at the Amelogenin locus recorded providing the fluorescence intensity measurements (error bars indicate standard deviation, $n = 3$).

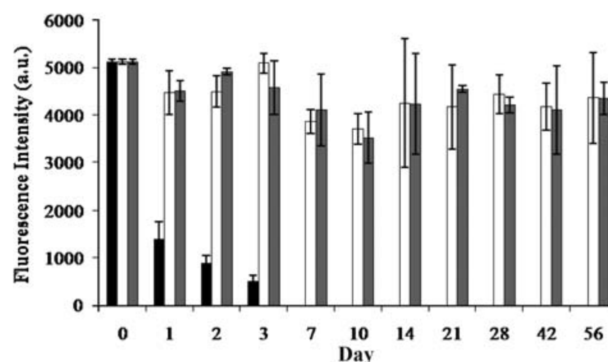

Fig. 6 Relative fluorescence intensities, as determined by capillary electrophoresis, from amplification of a single locus (Amelogenin) when PCR reagents (without DNA) were stored at different temperatures: room temperature (black bar), 4 °C (white bar) and −20 °C (grey bar) (error bars indicate standard deviation, $n = 3$).

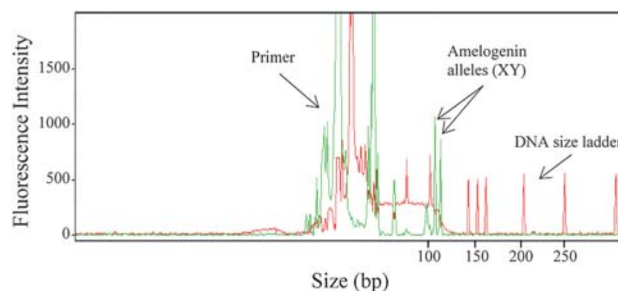

Fig. 7 Electropherogram showing PCR products from amplification of the Amelogenin locus ($X = 104$ bp and $Y = 110$ bp), using DNA extracted from a male volunteer, on the 'ready-to-use' microfluidic device as confirmed on an ABI Prism 310 Genetic Analyser.

Conclusion

This paper reports the successful integration of direct DNA extraction and PCR amplification on a single, self-contained microfluidic device. All the necessary extraction and PCR reagents were pre-loaded onto the device in specific compartments and no external pumps were required as movement was achieved using EOP. In this novel system, a bi-functional silica monolith provided both a solid-phase for DNA extraction and a pump for EOP. An evaluation of the encapsulation of PCR reagents for storage on the microfluidic device showed a final concentration of 1.5% (w/v) agarose gel provided the greatest degree of reagent stability. The gel loaded with reagents could be stored at 4 °C for at least 8 weeks and no interference with the amplification process was observed. Storing the reagents on the device reduces the size of the system footprint by eliminating the need for any external reservoirs of solutions and their mechanical manipulation. The approach developed also serves to increase the reproducibility of the analysis process as any handling errors related to preparing reagents and flowing them into the device will be reduced.

Using reverse EOF in combination with electrophoretic flow improved DNA extraction efficiencies. The use of EOP also resulted in an effective pumping method for performing DNA extraction and amplification, eliminating the need for complex

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mechanical interfaces. It has been demonstrated, by successful amplification of the target locus from the eluted DNA, that no detrimental electrophoretic effects on the PCR reagents are observed. By reducing user intervention and simplifying the pumping interface the proposed system exhibits reduced potential for introducing contamination, a particularly important consideration when dealing with clinical or forensic samples. In conclusion, the work presented here offers the potential for creating 'ready-to-use' microfluidic devices that can be used in fully automated systems for rapid sample processing. By combining the current methodology with PCR product detection, *via* real-time PCR or capillary electrophoresis, on a single device a complete "sample in-answer out" system could be generated.

Acknowledgements

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