THE UNIVERSITY OF HULL

Integrated DNA Extraction and Amplification on a Microfluidic Device

being a Thesis submitted for the Degree of

Doctor of Philosophy

in the University of Hull

by

Kirsty Jane Shaw

BSc (Imperial College, London)
MSc (King's College, London)

September 2009

Abstract

An evaluation of DNA extraction and amplification performed in microfluidic systems was carried out, with the aim of integrating the two processes in a single microfluidic device. This integrated device will then be incorporated upstream of capillary gel electrophoresis and fluorescence-based detection for development of a completely integrated genetic analysis system.

DNA extraction was performed using a silica substrate with both hydrodynamic and electro-osmotic pumping (EOP), resulting in maximum DNA extraction efficiencies of 82% and 52% respectively under optimised conditions. While the DNA extraction efficiency was lower using EOP, this method eliminates the need for external pumps and ensures easier mechanical connection to the microfluidic device. The use of thermally activated silica monoliths as the solid-phase resulted in superior DNA extraction efficiencies compared to when photo-initiated monoliths and silica beads were used.

DNA amplification of up to nine forensically relevant loci was successfully achieved on the microfluidic device in volumes as low as 1.1 µl using Peltier heating. A combination of silanisation and dynamic passivation was required to prevent PCR inhibition resulting from DNA polymerase adsorption. A custom-built microwave heating system was also evaluated, which was capable of heating and cooling rates of 65°C/second and 58°C/second, respectively.

EOP was used in the generation of an integrated microfluidic device, for DNA extraction and amplification. The silica monolith used as the solid-phase for DNA extraction also acted as a pump for electrokinetic movement. All necessary reagents for carrying out both DNA extraction and amplification were encapsulated in agarose gel and pre-loaded onto the microfluidic device creating a self-contained, ready-to-use system. Following addition of the biological sample to the microfluidic device, all electrokinetic movement and thermal cycling was controlled using a custom-built operating system.

Acknowledgements

I would like express my thanks to my supervisors Professor Steve Haswell and Dr. Charlotte Dyer for all their help and guidance through my studies. I am very grateful to Dr. Peter Docker and Dr. Domino Joyce for all their support and willingness to share their expertise.

I would also like to thank the rest of the 'At scene of crime' group namely Professor John Greenman, Professor Gillian Greenway, Miss Jennifer Oakley and our external collaborators: John Yelland, the Centre for Integrated Photonics, the Forensic Science Service and JLS Designs Ltd.

I am grateful to Miss Lauren Thain and Miss Elizabeth Hughes, supervised undergraduate project students, whose fruitful preliminary work formed the basis of the further investigation carried out here. For technical assistance, I would like to acknowledge Dr. Steve Clark for microfluidic device manufacture and Mr. Tony Sinclair for production of the monolith SEM images.

I am especially thankful to my family for all their love and support throughout my studies in particular my mum, my sister and my wonderful husband, Mike.

Abbreviations

BCA	- bicinchoninic acid
bp	- base pairs
BSA	- bovine serum albumin
CD	- compact disc
CJ	- criminal justice
DNA	- deoxyribonucleic acid
dNTP	- deoxyribonucleotide triphosphate
dsDNA	- double stranded DNA
DTT	- dithiothreitol
EDTA	- ethylenediaminetetraacetic acid
EOF	- electro osmotic flow
EOP	- electro osmotic pumping
EPDMA	- epoxy (poly)dimethylacrylamide
FSS	- forensic science service
GAPDH	- glyceraldehyde-3-phosphate dehydrogenase
gDNA	- genomic DNA
kbp	- kilobasepairs
LMT	- low melting temperature
MPTMS	- methacryloxypropyltrimethoxysilane
NDNAD	- national DNA database
OTS	- octadecyltrichlorosilane
PCR	- polymerase chain reaction
PDMS	- poly(dimethylsiloxane)
PEEK	- poly(etheretherketone)
PEG	- poly(ethylene glycol)
PMMA	- poly(methyl methacrylate)
POP4	- performance optimised polymer 4

ProK	- proteinase K
PVP	- poly(vinylpyrrolidone)
RF	- radiofrequency
RFLP	- restriction fragment length polymorphism
RNA	- ribonucleic acid
RT-PCR	- reverse transcription – polymerase chain reaction
SEM	- scanning electron microscopy
SGM	- second generation multiplex
SNP	- single nucleotide polymorphism
SPE	- solid phase extraction
ssDNA	- single stranded DNA
STR	- short tandem repeat
TE	- tris/EDTA
TEOS	- tetraethyl orthosilcate
TMOS	- tetramethyl orthosilicate
TPS	- trichloro((1*H*,1*H*,2*H*,2*H*) perfluorooctyl)silane
TWTA	- travelling wave tube amplifier
μTAS	- micro total analysis system
UV	- ultra violet

Table of Contents

List of Figures

List of Tables

List of Equations

1 Introduction

Since the discovery of the structure of deoxyribonucleic acid (DNA) by James Watson and Francis Crick over 50 years ago significant advances have been made in genetic analysis. From simple ABO blood typing to complete genome sequencing, human genetic analysis can now be used to provide important information about individuals. In medical diagnostics, for example, DNA analysis can be used for the detection of inherited genetic conditions such as Huntington's disease.[1]

DNA analysis of biological samples has found also widespread application in the field of forensic science. The seminal work of Alec Jeffreys in 1985 on DNA fingerprinting provided a valuable tool for human identity testing. Over the past two decades, substantial progress has been made on increasing the power of discrimination of DNA profiling techniques for human identification. In particular, there has been a significant impact in the UK, as it was the first country in the world to establish a national DNA database (NDNAD) which was set up in 1995.[2] At present the NDNAD is the largest of any country with approximately 5.2% of the UK population on the database.[3] Biological samples sent to the forensic laboratory can be broadly divided into two categories: criminal justice (CJ) samples and crime scene samples, the DNA profiles from which are loaded onto the NDNAD.

CJ samples are those which are taken as reference samples from suspects or victims and are most commonly a buccal swab used to collect cheek cells. As of March 2007, over 4.4 million such samples were retained on the NDNAD. Samples collected at the scene of a crime can take many different forms including cigarette butts or blood spatter. As of March 2007, 285,848 such crime scene samples were retained on the NDNAD, with 44,224 matches between suspects and crime scenes reported in 2006/2007.[4] Once the sample has reached the laboratory, the average turnaround time for sample processing is 3 days and the average cost of analysing a sample by DNA profiling is £35.

1.1 Genetic Analysis

DNA contains the genetic information that is passed down from parent to progeny. In 1953, Watson and Crick elucidated the structure of DNA and found it to be a double-stranded helix composed of two single-stranded oligonucleotide molecules hybridised together.[5] The biochemical structure consists of deoxyribonucleotides linked together by covalent phosphodiester bonds.

Each deoxyribonucleotide is composed of a deoxyribose sugar, a phosphate group and a nitrogenous base.[6] The base can be either a purine, adenine (A) or guanine (G), or a pyrimidine, cytosine (C) or thymine (T). Hybridisation between the two DNA strands is facilitated by hydrogen bonding between complementary bases on opposing strands. Specifically, A pairs with T through two hydrogen bonds and G pairs with C through three hydrogen bonds (Figure 1.1). The melting temperature of the DNA sequence, an important factor in DNA annealing and denaturation, is dependent upon the relative composition of A/T and G/C bases which determines the thermal energy required to break the hydrogen bonds present.

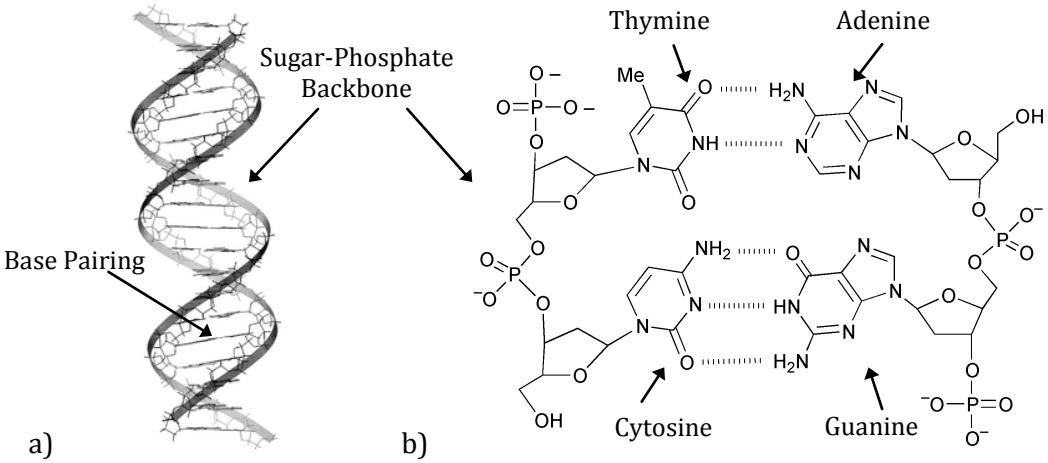


Figure 1.1: a) Double-stranded DNA helix, b) Individual base pair structure showing hydrogen bond formation during hybridisation. Adapted from Belmont *et al.*[7]

Human genomic DNA is packaged into the nucleus of cells in the form of chromosomes, of which there are 46 (23 pairs) in all nucleated cells apart from gametes. The ova and spermatozoa contain only 23 single chromosomes each, and during the process of fertilisation the male and female chromosomes combine to give the full complement of DNA in the resultant cell, with one chromosome from each pair inherited from each parent. Mitotic cell division then ensures that the DNA from both parents is present in the correct amounts in all cells in the foetus (Figure 1.2).

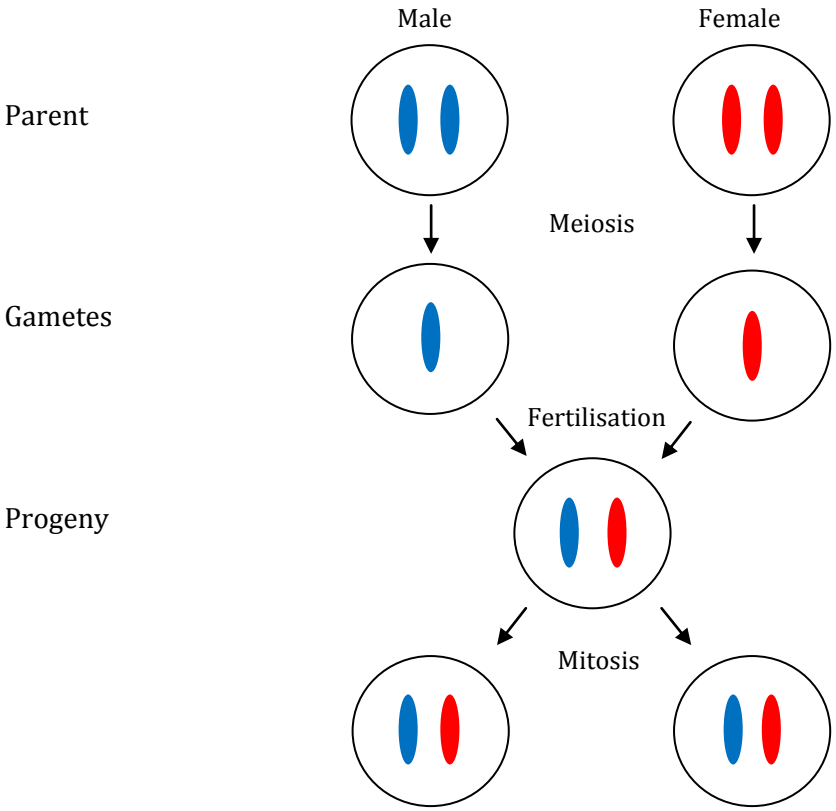


Figure 1.2: Schematic representation of the transfer of genetic material through inheritance.

Within the chromosome, the DNA sequence comprises protein coding and non-coding regions, of which the coding regions are termed genes. Genes in turn are made up of exons, which constitute the protein coding sequences and introns, which are the

intervening non-coding sequences. Within the non-coding regions of DNA variation markers, known as polymorphisms, can be found. Commonly, these are variations, between different individuals, in either sequence or length of DNA sequence at a specific site within the chromosome (Figure 1.3).

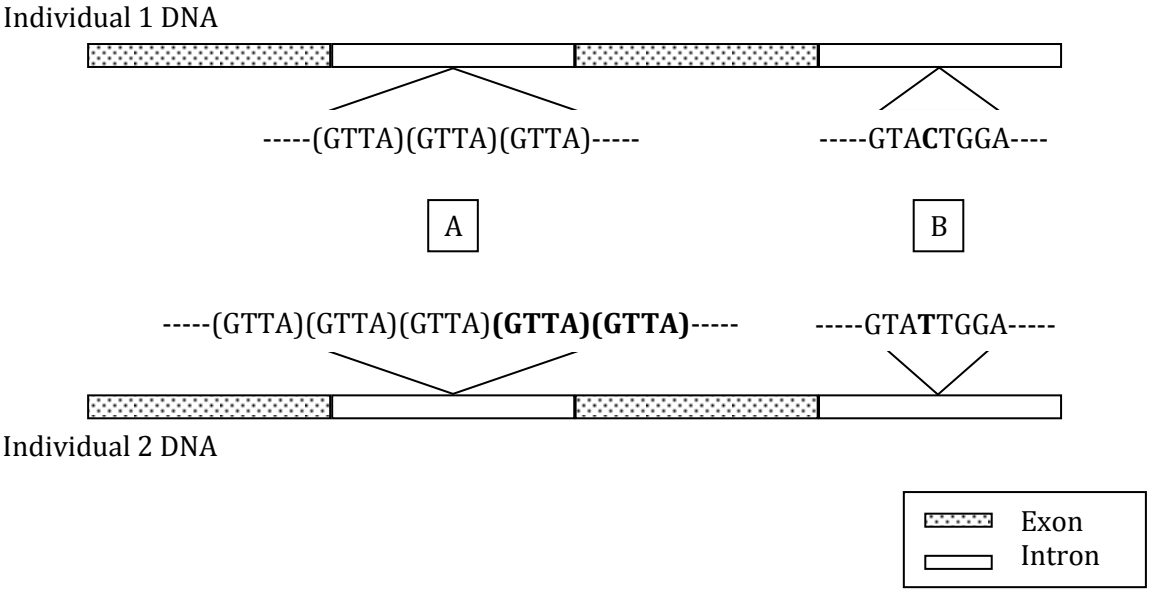


Figure 1.3: Diagram showing examples of A) sequence length and B) specific nucleotide sequence polymorphisms, demonstrating variation occurring within non-coding (intronic) regions of DNA between difference individuals.

The location of these sequence variation markers is termed a locus and the different polymorphisms possible at this location termed alleles. If the alleles at a given locus are the same on both chromosomes, then the genotype is described as homozygous whereas if they are different then the genotype is said to be heterozygous. Different individuals will have different alleles present at each given loci. Analysis of the alleles present at a single locus defines an individual's genotype and if multiple loci are examined then this information can be used to build up a DNA profile of an individual.

Using population genetics, the frequency of each possible allele occurring at a particular locus can be calculated for different races, permitting the determination of the probability of two individuals having the same alleles at that locus. If multiple loci, which are located on different chromosomes and therefore inherited independently during meiosis, are analysed this provides a greater degree of discrimination as the product rule can be applied. The product rule is whereby the genotype frequencies for each locus are multiplied together.[2] For example:

If a person is blood type A (allele frequency = 0.42) and rhesus negative (allele frequency = 0.17) then by applying the product rule (0.42 x 0.17), the likelihood of another individual having the same phenotype i.e. also being blood type A and rhesus negative, is 0.07 or 7%.[8]

1.1.1 Techniques for Genetic Analysis

A wide range of techniques are available for performing genetic analysis allowing the distinction between individuals based on their genetic properties. The ABO and Rhesus blood grouping systems are a commonly used example which classifies or 'types' an individual's blood based on inherited genetic properties.[2] While blood typing can be performed rapidly, it does not provide a very good degree of discrimination, for example 40% of the population is type O and therefore share the same genetic signature with reference to this particular locus.

In 1985, Alec Jeffreys developed a method of genetic profiling known as DNA fingerprinting, which significantly increased the discriminatory power of genetic analysis methods.[9] DNA fingerprinting was originally performed using restriction fragment length polymorphism (RFLP) analysis. RFLP analysis is carried out using restriction enzymes which cut the DNA at a specific recognition sequence, for example the endonuclease *Eco*R1 cleaves DNA at the sequence motif 5'-GAATTC-3'.[10] The number and location of such cleavage sites varies between individuals and therefore results in the production of

different size DNA fragments. These restriction digest reaction products can then be separated by size, using gel electrophoresis, resulting in a specific band pattern for each individual. However, RFLP analysis is a time consuming process and requires relatively large amounts of DNA (~6µg).[11] In addition the technique is not ideally suited to automation or the simultaneous analysis of multiple loci.[2] In order to increase the degree of discrimination between individuals, DNA analysis moved from analysing RFLPs to short tandem repeats (STRs) in genomic DNA which forms the basis of DNA profiling (Chapter 1.1.2).

As well as differences in length, sequence polymorphisms i.e. variation in the nucleotide sequence, are also commonly studied. By directly sequencing genomic DNA, single nucleotide polymorphisms (SNPs) between individuals can be detected.[2] Additionally, in cases where the genomic DNA is severely degraded or maternal relationships are important then mitochondrial DNA, present in much higher copy numbers per cell, can be sequenced providing valuable information.[2]

1.1.2 DNA Profiling

STRs are length polymorphisms similar to those found exploited in RFLP analysis but the repeat sequence is shorter in length, usually four base pairs long, known as a tetrameric repeat. The nomenclature for STR markers is dependent upon whether they are located within or outside of a gene. For those STR markers present within a gene, the gene name in used for the designation, e.g. TH01 is located within the **t**yrosine **h**ydroxylase gene in intron **01**. Those STR markers located outside of a gene are given a designation based upon their chromosomal location. For example, D16 S539 is so named as it is a **D**NA marker, located on chromosome **16**, present as a **s**ingle copy sequence and was the **539**th locus to be described on that chromosome.[2]

Each allele, at a given locus, is defined by the number of repeat sequences it contains e.g. Allele 10 at TH01 would mean there were 10 repeats of the sequence AATG at the TH01 locus. In addition there are a number of microvariant alleles which do not contain full repeat units, such as allele 9.3 at the TH01 locus which contains 9 tetrameric repeats units and one 3 base pair unit due to a single base pair deletion.[2]

DNA profiling, based on STR analysis can be performed on a multitude of biological sample types including blood, saliva, sweat, semen, hair, tissue and bones. The first commercially available multiplex STR kit was produced by Promega in 1994 and consisted of three loci (CSF1PO, TPOX and TH01) with a discrimination potential of around 1 in 500.[2] Over the years more loci have been added to such DNA profiling kits to increase the discrimination potential of the systems. At present forensic companies in the United Kingdom (UK), including the Forensic Science Service (FSS), use the AmpF*l*STR® Second Generation Multiplex (SGM) Plus™ PCR Amplification kit [Applied Biosystems, UK], which analyses ten individual STR loci (D3 S1358, vWA, D16 S539, D2 S1338, D8 S1179, D21 S11, D18 S51, D19 S433, TH01 and FGA) plus the Amelogenin sex marker, details of which are given in Table 2.3.[12] An example DNA profile produced using the AmpF*l*STR® SGMPlus™ kit, in the form of an electropherogram, is shown in Figure 1.4. The discrimination potential of this system is greater than one in a billion. STR analysis is also used for paternity testing, whereby the child's profile can be compared to potential fathers.[2] As with forensic cases, samples can be excluded with great certainty, whereas inclusion is based on probability.

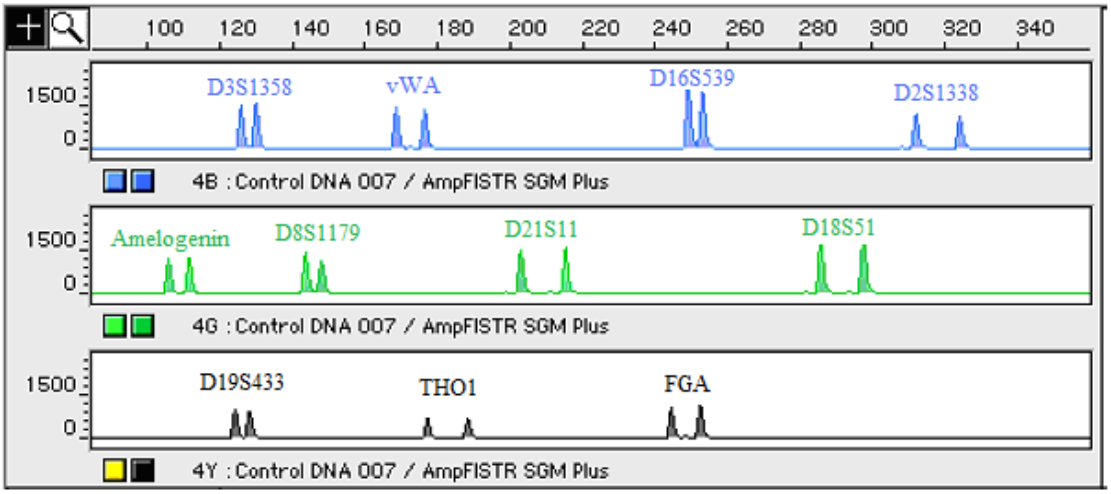


Figure 1.4: Example electropherogram of alleles amplified using the AmpF*l*STR® SGMPlus™ kit [Applied Biosystems, UK].[12] Fluorescence intensity is shown on the y axis and size (in base pairs) is indicated on the x axis. Three different fluorescent tags are used to allow multiplex analysis of loci permitting differentiation of several amplicons of a similar size range. By including a DNA size ladder during the analysis, designation of the alleles can take place based on their size.

There are 4 main steps involved in the DNA profiling process following biological sample collection: cell lysis and DNA extraction, DNA amplification, electrophoretic separation and fluorescent detection of amplified products (Figure 1.5). The principles behind these techniques on the macro-scale will be discussed below followed by current miniaturisation efforts with the focus on DNA extraction (Chapter 1.3) and amplification (Chapter 1.4) procedures.

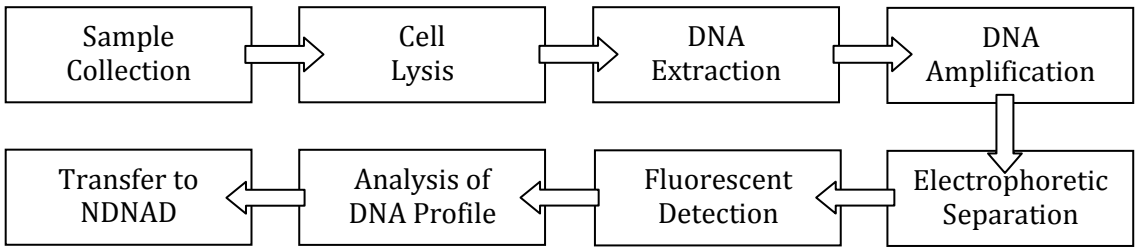


Figure 1.5: Flow diagram showing the various stages of DNA profiling.

1.2 Microfluidics

Microfluidics is a term used to describe the manipulation of liquids within an environment with micron dimensions or microlitre volumes. The rapid expansion of the field of microfluidics over the past decade has been driven by the requirement for multiple process to be integrated together on a single microfluidic device, so called micro-total-analysis (μTAS) or Lab-on-a-Chip systems.[13]

Scaling laws mean that at the microfluidic level, volume forces become largely unimportant and surface forces become dominant. For example, a linear reduction of a factor of 10^3 results in a factor of 10^9 volume reduction.[14] The increased surface area to volume ratio characteristic of microfluidic systems, can have a significant effect on (bio)chemical reactions, for example adsorption of DNA polymerase required for the polymerase chain reaction (PCR) occurs if the internal surfaces of the microfluidic device are not coated leading to inhibition of the DNA amplification reaction.[15]

The flow of fluids within microfluidic systems can be examined by looking at the Reynolds number (R_e):

$$R_e = \frac{\rho d \nu}{\eta} \qquad \textbf{(Equation 1.1)}$$

where ρ is the density of the liquid, d is the diameter of the channel, ν is the average velocity of the moving liquid and η is viscosity of the liquid. An R_e value >2300 is indicative of turbulent flow, whereas an R_e value <2000 indicates laminar flow. Due to the small dimensions present in microfluidic devices, it is laminar flow regimes which dominate.[16]

Transport within microfluidic systems can be controlled mechanically, using a pump, electrically, using an applied voltage, or by diffusion. The diffusion distance, x, of a molecule is governed by the Einstein-Smoluchowski equation:

$$x = \sqrt{2Dt} \qquad \textbf{(Equation 1.2)}$$

where D is the diffusion constant for a given molecule and *t* is time. Diffusive mixing can occur rapidly in microfluidic system, for example, the mixing time of a dye in water based purely on diffusion is 10^5 seconds in a system of 10 cm compared to 10 seconds in a system of 100 μm.[13]

The miniaturisation of (bio)chemical processes by utilising a microfluidic platform has many inherent advantages. A reduction in the amount of sample and reagents required can reduce associated costs. Speed of analysis is also increased and can be further enhanced by using parallel processing. The possibility of mass production of microfluidic devices leads to the potential for cheap, disposable Lab-on-a-Chip systems [14] and the use of microfluidic technology has enabled the possibility of producing fully portable systems to allow point-of-care analysis.[15]

1.2.1 Material Selection for Microfluidic Device Fabrication

The choice of substrate material for production of the microfluidic device is crucial for optimum performance. In the development of integrated microfluidic systems, a compromise is often required as some processes will perform better in one substrate than another. As no one substrate will be able to provide optimal performance characteristics in all areas e.g. cost, biocompatibility and optical transparency, the most appropriate material for the techniques to be performed on the microfluidic device must be chosen.

Both silicon and glass have been widely used in the manufacture of microfluidic devices. For example, silicon has high thermal conductivity enabling fast temperature ramping

which is ideal for DNA amplification but bare silicon inhibits PCR and therefore surface coating is required prior to use. Silicon is not optically transparent and does not support electro-osmotic flow (EOF) therefore would not be suitable for capillary electrophoresis and fluorescence-based detection of the PCR products.[16] Another popular alternative is the use of glass, which has well defined surface chemistry, good EOF characteristics and optical transparency for detection.[15]

More recently the use of polymers has been investigated due to the low cost and ease of fabrication to aid the development of single-use, disposable microfluidic devices which can be mass produced. A wide range of polymers exist with differing properties which can be exploited depending on the intended application. Poly(dimethylsiloxane) (PDMS) is an example of an inexpensive polymer which exhibits good biocompatibility and optical transparency. However, its permeable nature means that sample loss can occur, e.g. during thermal cycling for PCR. Another example is the use of poly(methyl methacrylate) (PMMA) for microfluidic device, which can be manufactured in minutes using CO_2 laser ablation. PMMA also has good biocompatibility and optical transparency but is less suited to the high temperatures required for PCR thermal cycling.[17, 18] Hybrid devices can be used, e.g. PDMS-glass, which enable the favourable properties of each component to be exploited.[16]

1.2.2 Movement of Reagents

Movement of reagents around microfluidic systems can be achieved using a variety of different means, including hydrodynamic pumping, EOF and centrifugal forces. Centrifugal forces have been demonstrated relatively recently for use on miniaturised systems and rely on the use of a compact disc (CD) style footprint in which solutions are radiated outwards from central reservoirs, through features on the microfluidic device, as the CD is spun.[19] Hydrodynamic pumping, is a well established methodology in which

reagent movement is achieved using the attachment of syringes, through tubing and connectors, to the microfluidic device. The use of positive or negative pressure creates a parabolic flow profile within the channel which means that the flow of solutions is faster within the centre of the channel due to frictional forces at the surface (Figure 1.6a).

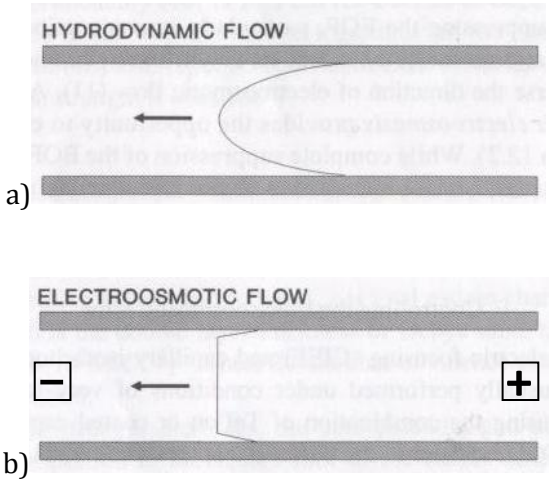


Figure 1.6: Comparison of the flow profiles for movement of solutions within microfluidic devices produced when using a) hydrodynamic pumping or b) electro-osmotic flow.[20] Note the parabolic flow profile for hydrodynamic pumping compared to the relatively flat flow profile produced when electro-osmotic flow is used to create bulk movement of solutions towards the cathode.

Electro-osmotic flow (EOF) describes the bulk movement of solutions in the presence of an applied electric field. An electrical double layer is created at the surface by the electrostatic attraction of cations to the deprotonated silanol groups on the internal glass surface. The inner cation layer is a tightly held fixed layer but is not dense enough to neutralise all the negative charges at the surface, therefore a second outer layer of cations forms producing a diffuse mobile layer. It is this mobile layer which is pulled towards the cathode, in the presence of an applied electric field, dragging with it the bulk solution (Figure 1.7).[21] In the absence of pressure differences across the length of a microfluidic channel, EOF has a flat flow profile compared to the parabolic one produced by

hydrodynamic flow which means that all molecules exhibit the same velocity, except for those very close to the internal surface wall (Figure 1.6b). Electro-osmotic pumping (EOP) can be achieved by performing EOF across a porous support which prevents back flow of solutions.

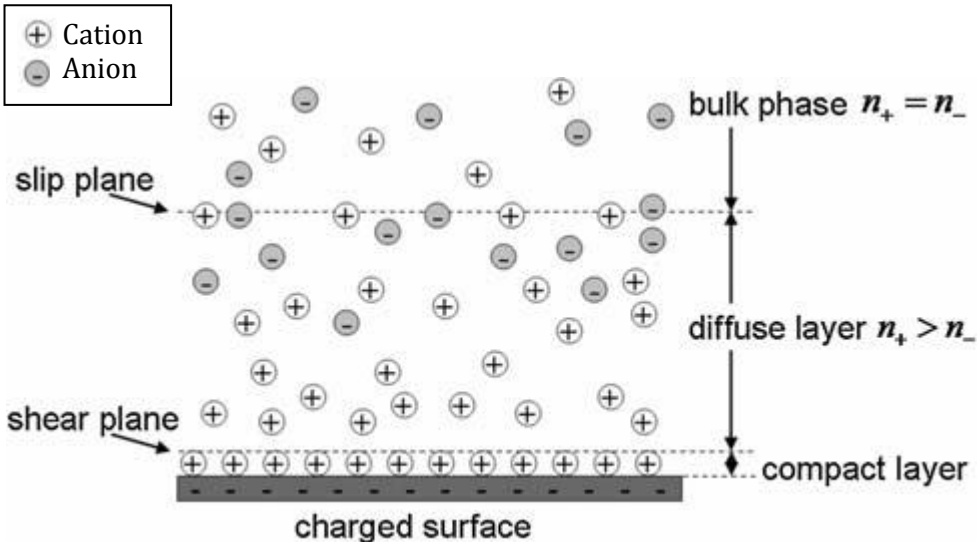


Figure 1.7: Diagram showing the electrical double layer at a charged surface which results in electro-osmotic flow in an applied electric field.[22]

1.3 DNA Extraction

1.3.1 Traditional Sample Preparation and Cell Lysis

Cell lysis is essential for the release of the DNA contained within the cell prior to analysis, with DNA profiling specifically focusing on genomic DNA (gDNA) contained within the cell nucleus. Cell lysis can be achieved in a variety of ways both mechanical and chemical. Mechanical cell lysis methods include sonication, heating, electrical stimulation[23] and laser ablation.[19]

Chemical cell lysis can be achieved through the addition of chaotropic agents such as guanidine hydrochloride, which not only serve to lyse the cells but also inactivate nucleases which would otherwise go on to degrade the released DNA.[24] Cell lysis can be aided by the use of surfactants, such as sodium dodecyl sulphate or Triton X-100 that, due to their amphoteric nature, dissolve lipids present in the cell wall.[25] However, surfactants can interfere with downstream applications such as PCR therefore limiting their usage. In addition, proteinase K (ProK) is often added to enzymatically digest the proteins which protect the DNA whilst in chromosomes, but this requires subsequent heat deactivation as ProK is also a known inhibitor of PCR.[26]

Chemical cell lysis methodologies can also be exploited to separate mixed cell types such as in sexual assault case samples which require the separation of sperm and epithelial cells. This is commonly achieved using differential extraction, which exploits the different biochemical properties of the two cell types. Sperm cells have a 'harder' nuclear membrane rich in disulphide bonds making them resistant to standard cell lysis conditions.[2] This allows epithelial cells to be lysed and their DNA extracted whilst the sperm cells remain intact. Subsequent addition of a reducing agent, such as dithiothreitol, enables lysis of the sperm cells facilitating extraction of the male DNA.

1.3.2 Miniaturised Sample Preparation and Cell Lysis

As well as the adaptation of traditional sample preparation and cell lysis techniques, described above, for microfluidic applications, novel techniques have also been developed which favour the conditions present in a microfluidic environment. When dealing with crude biological samples it is important to isolate the correct cell types for analysis. For example, when using whole blood it is important to isolate only the leucocytes (white blood cells) as, unlike erythrocytes (red blood cells), they contain DNA. Microfilters can be used to capture the larger leucocytes (6 – 10 μm) whilst letting the smaller erythrocytes pass unhindered.[27] Furthermore filters can be modified to include, for example, immobilised antibodies and therefore capture specific cell types whilst allowing unwanted cells to flow through.[28]

Different cell types can also be separated by exploiting their different physical properties, as demonstrated by Horsman *et al.* using a microfluidic device for the separation of sperm and epithelial cells.[29] When a mixed sample is added to an inlet reservoir the larger epithelial cells (diameter 40-60 μm) sink to the bottom and adhere, via cell surface binding proteins, to the glass surface. By applying a low flow rate, the smaller sperm cells (4-6 μm head diameter) can be separated off while the epithelial cells remained adhered to the glass surface allowing subsequent analysis of the separated fractions by conventional laboratory methods (Figure 1.8).

Traditional chemical cell lysis techniques (Chapter 1.3.1) have been directly applied to microfluidic systems including the use of chaotropic agents such as guanidine thiocyanate[25, 30] and guanidine hydrochloride,[31] surfactants such as Triton X-100[25, 31] or SDS[30, 32] and reducing agents, such as dithiothreitol (DTT), for lysis of sperm cells.[31] Cell lysis can also be achieved by modification of extracellular osmolarity resulting in hypoosmotic shock causing swelling and ultimately lysis of the cells.[33]

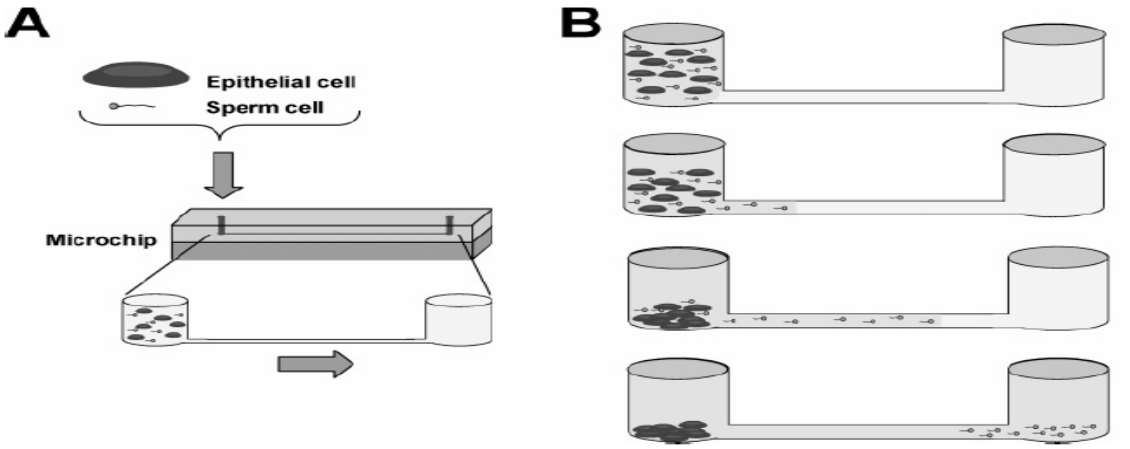


Figure 1.8: Schematic showing the separation of sperm and epithelial cells based on the adhesion of epithelial cells within a microfluidic device and subsequent flow driven separation of sperm cells from the mixed sample.[29]

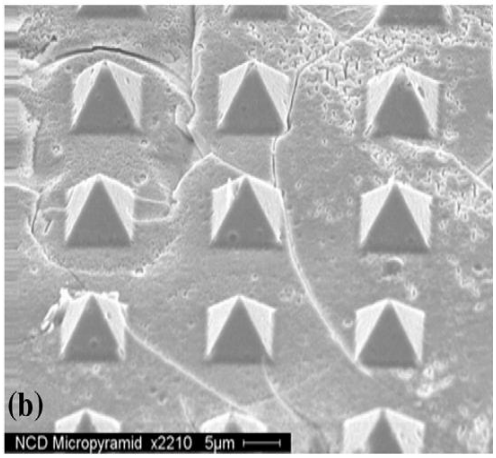
Mechanical cell lysis methods have also been shown to be effective in a microfluidic environment. Thermal shock, leading to cell lysis, can be achieved using a two minute hold at 94°C immediately prior to PCR, yielding sufficient DNA for amplification.[34] Kim *et al*., demonstrated that by incubating cells and beads in a microchamber on a compact disc and applying centrifugal force, cell lysis could be achieved due to the collisional and frictional forces created by the presence of the beads. This resulted in a 65% lysis efficiency compared with conventional surfactant-based methods.[35] Using a microbeam laser to deliver a highly focused pulse can also be used to initiate cell lysis [36].

Electroporation is a technique which has been incorporated into a number of microfluidic devices.[37-39] It involves the application of an electric field to a cell resulting in the creation of micropores thus increasing the permeability of the cell. By removing the electric field the micropores can re-seal, however, if the electric field applied is too high then irreversible damage to the cell membrane occurs resulting in lysis.[37]

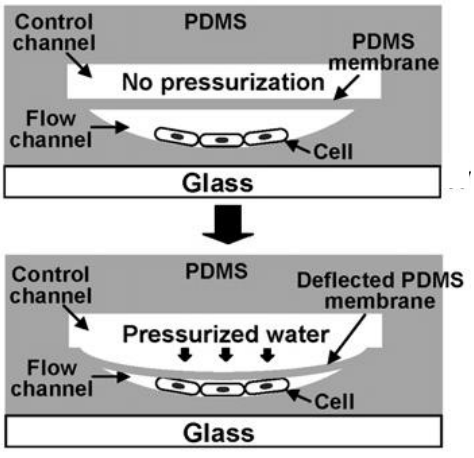
Ultrasonication is a powerful technique which utilises sound energy to disrupt cellular membranes resulting in cell lysis. However, power limitations in a microfluidic format can

reduce the efficiency of the process. Khanna *et al.,* have showed that by including micro-spikes (Figure 1.9a) in the cell chamber the efficiency of the cell lysis procedure using ultrasonication on a microfluidic device can be increased by 400%.[40] The use of nanoscale barbs for cell lysis in a microfluidic environment, without the use of ultrasonication, has also been demonstrated to be successful.[41]

In addition, the use of miniaturised systems has lead to the development of novel techniques for cell lysis within a microfluidic environment. Kim *et al.,* have developed a microfluidic device in which compressive force is applied to the cells through a deformable PDMS membrane (Figure 1.9b). Cell lysis was achieved by applying a pressure of 35kPa; below this pressure the system could also be used for monitoring mechanically induced cell stimulation.[42] Microelectrodes can also be used to generate localized hydroxide ions which cause cell lysis, with the added advantage that the hydroxide ions react with hydrogen ions present in the system resulting in a neutral pH, important for downstream applications.[43]



a)



b)

Figure 1.9: Mechanical methods of cell lysis in microfluidic devices showing examples of a) an electron micrograph of a micro-spike used to mechanically aid sonication[40] and b) schematic showing demonstrating the use of compressive forces.[42]

1.3.3 Traditional Solid-Phase DNA Extraction

The isolation of DNA from biological samples constitutes the first step in a variety of bioanalytical techniques, for example PCR which performs optimally when using purified DNA free from potential inhibitors of the amplification reaction. A wide variety of methods exist for performing DNA extraction from biological samples of which some examples are given below (Table 1.1).

Extraction Method	Principle	Advantages	Disadvantages
Chelex™ [2]	A chelating resin which binds polyvalent nuclease co-factors, such as Mg^{2+}, protecting the DNA from digestion.	Quick, small number of steps carried out in a single tube reducing the risk of contamination.	Produces ssDNA therefore only compatible with PCR-based analysis.
Phenol-Chloroform[2]	Using a phenol-chloroform solution, DNA and proteins are separated as DNA is more soluble in the aqueous phase.	Most effective technique for extraction of high molecular weight DNA.	Time consuming, requires the use of hazardous reagents.
Ion Exchange[44, 45]	Anionic DNA is captured on a positively charged column, e.g. those with diethylaminoethyl groups. Altering the pH and salt concentration of the buffers used controls DNA binding, wash stringency and elution.	High degree of control during adsorption and elution steps allowing high specificity.	Requires alcohol precipitation of the DNA after elution.
Solid-Phase Silica[24]	DNA is adsorbed onto a silica solid support, potential contaminants are removed by washing and the purified DNA eluted from the support.	Allows pre-concentration of DNA sample.	Cannot distinguish between different types of nucleic acid.

Table 1.1: Examples of commonly used DNA extraction methods outlining the principles of each technique.

As biological samples in forensic investigations can be limited in terms of both quantity and quality, retrieving the maximum amount of DNA possible from the original sample is crucial. Therefore the use of solid-phase extraction (SPE) methodology is ideally applicable to such circumstances. One example of solid-phase DNA extraction methodology, used in many commercially available DNA extraction kits such as the QIAamp® DNA Micro Kit [Qiagen, UK],[46] involves the use of silica as the solid-phase to which the DNA binds in the presence of chaotropic agents, such as guanidine hydrochloride (GuHCl).

DNA adsorption is driven by the shielding of intermolecular electrostatic forces, the dehydration of both DNA and silica surfaces and the formation of intermolecular hydrogen bonds in the DNA-silica contact layer.[47] The DNA adsorption reaction can be described by (Equation 1.1):

DNA (hydrated) + silica (hydrated) + counterions $\rightleftarrows$ neutral DNA/silica complex + water

(Equation 1.3)[47]

In order to facilitate DNA adsorption to the silica surface, the electrostatic repulsion between the two anionic species must be overcome. In a high ionic strength solution the negative potential at the silica surface is reduced allowing dehydration and hydrogen bond formation to become the dominant forces. By using a high ionic strength solution which has a pH below that of the pK_a of the silica, electrostatic repulsion can be further reduced as protonation of the silica silanol groups is increased resulting in a decreased surface charge.[48] The chaotropic agent also forms hydrated ions by sequestering water molecules reducing the solvation of DNA and the silica surface, therefore driving the reaction to the right (Equation 1.1).[47] In addition, the chaotropic agent denatures the DNA allowing the exposed bases of the single-stranded (ss) DNA molecules to hydrogen bond with the silica surface.[48] Chaotropic agents also serve to lyse cells and inactivate nucleases which would otherwise go on to enzymatically digest the extracted DNA.[24]

With the DNA adsorbed onto the solid-phase, an alcohol wash can then be used to remove any cellular or proteinaceous debris which may interfere with downstream applications. The DNA can then be eluted from the solid-phase in a low ionic strength buffer (Figure 1.10).[48]

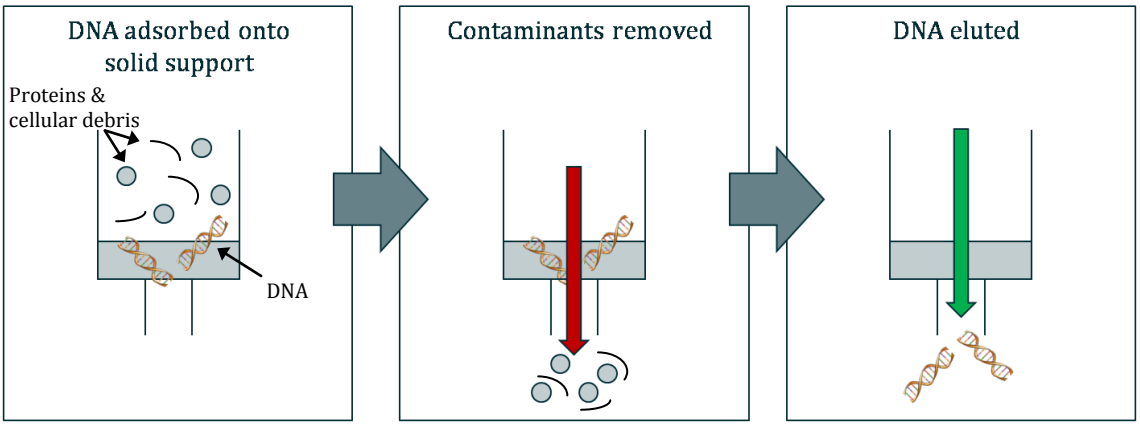


Figure 1.10: Schematic depicting the essential steps for solid-phase DNA extraction.

The surface of silica is made up of exposed siloxane bonds and silanol groups. Siloxane bonds (Si-O-Si) cannot form bonds with hydrogen donors but are hydrophobic and so can participate in reverse phase reactions that involve hydrophobic interactions such as protein binding. There are three types of silanol groups: isolated, vicinal and geminal (Figure 1.11). Tian *et al.*, demonstrated that silica with a higher concentration of free and vicinal silanol groups (Type A silica), is more effective at adsorbing negatively charged molecules. The more acidic silanols in Type A silica, exhibit increased protonation thereby reducing electrostatic repulsion of the DNA and enhancing binding.[48]

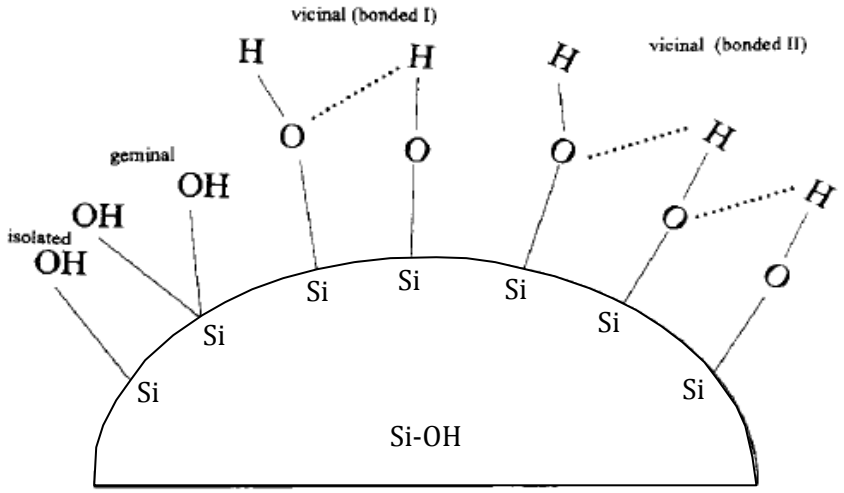


Figure 1.11: Schematic showing the different types of silanol groups present on the surface of silica.[49]

In order to improve the recovery of DNA during the extraction process carrier molecules such as ribonucleic acid (RNA),[50] salmon sperm DNA[51] or glycogen[52] can be used. As part of phenol-chloroform DNA extraction methodologies, glycogen can be used as a co-precipitant to increase the precipitation of DNA in the presence of alcohol. Carrier molecules have also been used in silica-based solid-phase DNA extraction procedures, for example, the addition of poly-A carrier RNA to the extraction matrix in commercially available Qiagen DNA extraction kits [Qiagen, UK] increases the amount of DNA recovered during the elution phase by an average of 24%.[53] It is hypothesised that on the silica matrix there are always a certain number of sites which will irreversibly bind nucleic acids. By including carrier RNA in the binding solution it can sacrificially bind to these sites and so the loss of important DNA is minimised leading to greater recoveries.[53] Poeckh *et al*., studied the adsorption isotherm behavior of DNA and RNA to silica beads in the presence of guanidine hydrochloride and showed that RNA adsorption was more efficient.[54]

1.3.4 Miniaturised Solid-Phase DNA Extraction

While a number of different solid-phases exist for DNA extraction, the focus here is on silica-based matrices which can take a variety of forms including microfabricated silica structures, silica microspheres (beads) and monoliths. The production of silica pillars during the fabrication of microfluidic devices offers probably one of the easiest methods for producing a solid support for DNA extraction with a large surface area to volume ratio (Figure 1.12). While the DNA extracted from such systems is of sufficient quality and integrity for PCR amplification, the efficiency of the process is very low. Initial studies using square pillars demonstrated DNA extraction efficiencies of between 10 and 20%, with either manual[55] or automatic control of reagents.[56] West *et al*., modelled an alternating array of teardrop-shaped pillars in order to create a more homogeneous flow distribution by the separation and recombination of flows. This increased the contact of DNA with the silica surface and enabled a DNA extraction efficiency of 25%.[57] Despite these improvements, the DNA extraction efficiency is still low and is not ideally suited to forensic work where often limited sample is available.

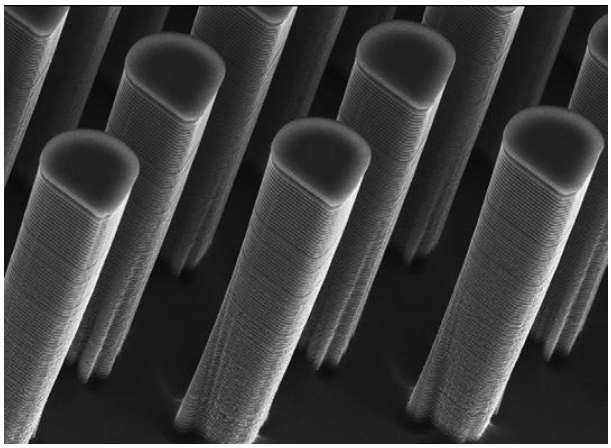


Figure 1.12: Electron micrograph showing silica pillars fabricated in a microfluidic device. Note the teardrop shape which facilitates production of a near homogeneous flow distribution facilitating DNA interaction with the pillar surface.[57]

The packing of silica particles ('beads') within a microfluidic device is one method by which a solid support for DNA extraction can be created. Silica beads (Figure 1.13a) can be either non-magnetic (pure silica particle) or magnetic (magnetic particle coated with silica), which affects how they can be applied to microfluidic systems. Non-magnetic silica beads can be contained within a microfluidic device either by trapping or immobilisation techniques. Trapping can be achieved using pillars [58] or dams [59] against which the beads can pack allowing solutions to flow through without displacing the beads. Alternatively tapered channel geometry can be used to hold beads in place by relying on the 'keystone' effect whereby the aggregation of a small number of initial beads at the taper forms a barrier against which beads subsequently added to the system can pack.[60] Immobilisation occurs when the surface chemistry of the beads and internal walls of the microfluidic device facilitates an interaction. Examples include the capture of streptavidin-coated beads on a biotinylated surface [61] and the use of poly(N-isopropylacrylamide)-coated beads which undergo a temperature sensitive reversible hydrophilic-hydrophobic transition resulting in immobilisation at higher temperatures (Figure 1.13b).[62]

Magnetic beads can not only be used to immobilise specific compounds based on their surface chemistry but can also act as maneuverable bio-molecule carriers. Different coatings can be applied to magnetic beads depending on their intended use; in this instance coating with silica enables DNA extraction to be performed.[63] Due to their magnetic properties such beads can be either held in place forming a plug, using an external magnet, while different reagents are flowed through the system and then the beads are released [64] or the beads themselves can be manipulated around the microfluidic systems through different reagents (Figure 1.13c).[63] When performing DNA extraction using magnetic beads, the purified DNA can either be eluted or the washed beads maneuvered directly into the PCR chamber for direct amplification, which will be discussed in more detail in Chapter 1.4.

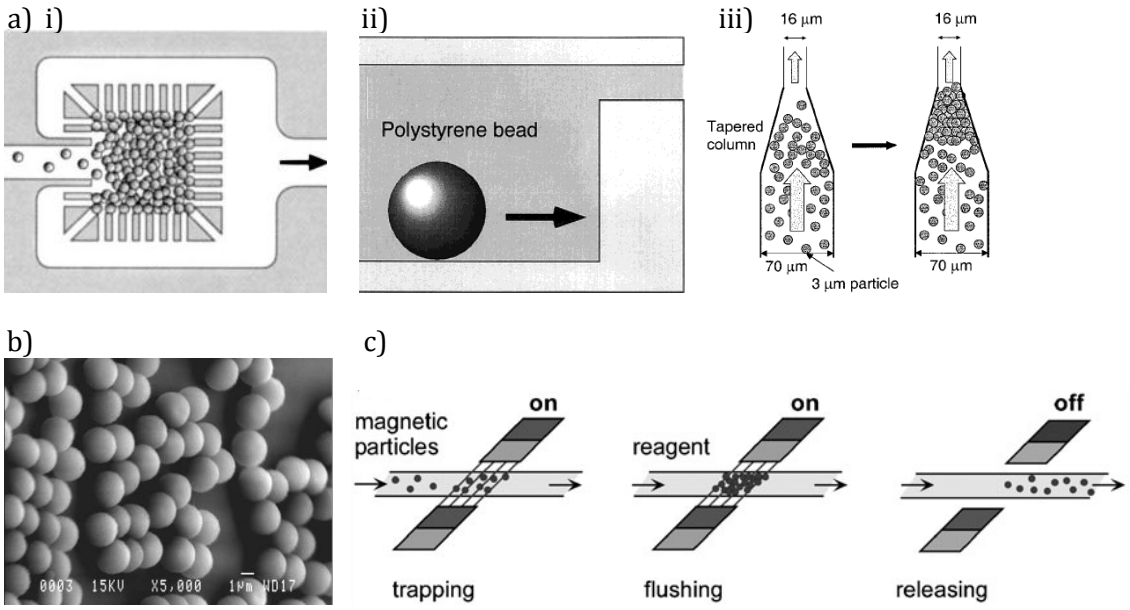


Figure 1.13: Schematic showing example techniques for retaining beads within microfluidic systems. Including a) trapping using i) pillars [58], ii) a dam [59] and iii) tapered channel geometry [60]; b) immobilisation using streptavidin-biotin binding [61] and c) trapping of magnetic particles.[64]

The efficiency of the DNA extraction process using silica beads is dependent on a wide range of factors including the size, shape and porosity of the silica beads. Tian *et al*., showed that irregular i.e. non-spherical, shaped silica beads produced more reproducible DNA extraction efficiencies due to the ease with which such beads could be packed within the microfluidic system.[48] For optimal DNA binding, a large surface area to volume ratio is required, which can be achieved by using either smaller or more porous beads although this can create problems with increased back pressure. Ji *et al*., investigated the effects of temperature on DNA extraction efficiencies and showed that optimum DNA binding occurs at 5°C, whereas optimum DNA elution occurs at 80°C.[27] DNA extraction efficiencies also vary depending on the starting material from which the DNA is to be extracted, for example, using pre-extracted DNA or whole blood as the starting material produced extraction efficiencies of 84% and 42% respectively.[65] This can be attributed to

competition for non-specific binding sites by other endogenous compounds found in complex biological samples.

Another important factor relating to DNA extraction using silica is the pH of the buffers used which greatly affect DNA adsorption and desorption. Geng *et al*., have shown that the pH of silica beads can be electrochemically altered using gold electrodes placed at either end of a packed silica bed. When a voltage is applied, the hydroxide ions generated at the cathode are swept over the packed bed causing an increase in pH and the subsequent release of adsorbed DNA from the surface of the silica.[66] The advantage of such a system is that adsorption and release of DNA can be controlled electrically eliminating the need for multiple solutions to be applied to the microfluidic device.

Despite the successes of applying silica beads to microfluidic systems to provide the solid-phase for DNA extraction there is a consistent lack of reproducibility throughout the literature.[48] For example, Wolfe *et al*., demonstrated a silica bead system with a DNA extraction efficiency of 57% but with a standard deviation of 43%.[67] In order to improve the reproducibility of miniaturised silica-based DNA extraction systems the use of sol-gels have been investigated. Sol-gels are defined as 'liquid colloidal suspensions which, when catalysed, gel to form solid structures', a methodology which is amenable to microfluidic systems.[67] Initial work using tetraethoxysilane-derived sol-gels, produced by thermal activation, demonstrated problems with small pore size and cracks in the structure formed as the sol-gel dries, resulting in poor DNA extraction efficiencies (maximum 33%). Nevertheless, further investigation combining the use of silica beads and sol-gels proved to be very successful. Production is a two-step process where silica beads are packed into the microfluidic device, restrained by a dam, and the sol-gel solution flowed over prior to catalysis. The resulting DNA extraction efficiency of the combined system was shown to be 71% ± 2%, with greater reproducibility than the silica beads alone.[67]

The same group have gone on to use this combined bead/sol-gel methodology to extract DNA for the identification of *Bacillus anthracis,* (the causative agent of anthrax), which

represents a significant biological threat, as demonstrated by its recent terrorist use when it was sent through the US postal service.[68] Furthermore this methodology has been used to perform DNA extraction from sperm cells, with the simple addition of DTT, which is required to reduce disulphide bonds prior to cell lysis, resulting in an average DNA extraction efficiency of 50%.[31] More recently, Kulinski *et al*., have used a combined silica bead/polymer sol-gel matrix to extract DNA from human urine samples which provide a significant challenge as small quantities of DNA are required to be extracted from relatively large sample volumes (Figure 1.14). While PCR-amplifiable DNA was recovered using the described methodology, no efficiencies for the recovery of DNA were given.[69]

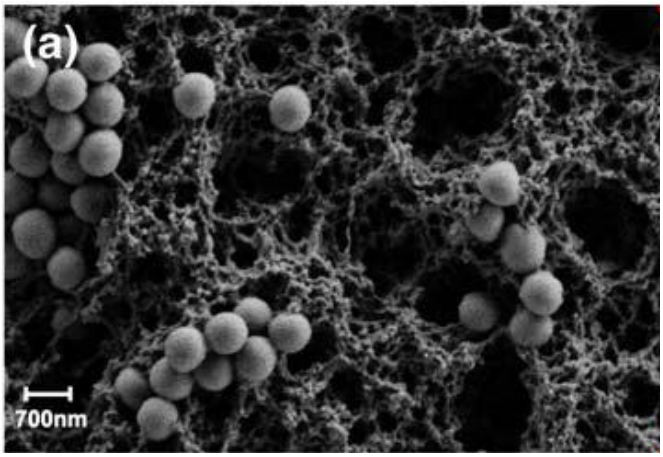


Figure 1.14: Scanning electron microscopy (SEM) image of combined bead/sol-gel monolith.[69]

While the use of bead/sol-gel methodology shows high DNA extraction efficiency and reproducibility, production is a multi-step, time-consuming process and so further research has been carried out using sol-gels (or monoliths) as stand-alone solid supports. Monoliths are described as a 'continuous mass of highly porous material, typically created by *in situ* polymerization of monomers and characterized by a bimodal pore structure, consisting of large flow-through pores for high permeability and small diffusion pores for the desired high surface area to provide a high capacity'.[70] Monoliths can be defined based on their porosity as microporous (<2nm pore size), mesoporous (2-50nm pore size) or

macroporous (>50nm pore size).[71] The choice of monomer is very important when producing a monolith capable of acting as the solid-phase for DNA extraction, for example the use of tetraethyl orthosilcate (TEOS) prevents extraction of PCR-amplifiable DNA and also has problems with high backpressure due to small pore sizes.[67] On the other hand the use of tetramethyl orthosilicate (TMOS) has been shown to create monoliths capable of DNA extraction from whole blood with efficiencies up to 70%.[72, 73] By including a porogen, poly(ethylene glycol) (PEG), in the reaction mixture the resulting monolith contains both macropores (created by the presence of PEG) and mesopores (which are intrinsic to a sol-gel) which creates a large surface area for DNA adsorption whilst allowing a good flow of solutions through the system. In addition, pre-treating the internal surface of the microfluidic device with sodium hydroxide (NaOH) allows it to participate in the condensation reaction, thus facilitating attachment of the monolith to the internal surface, reducing shrinkage of the monolith which is detrimental to performance (Figure 1.15).[70]

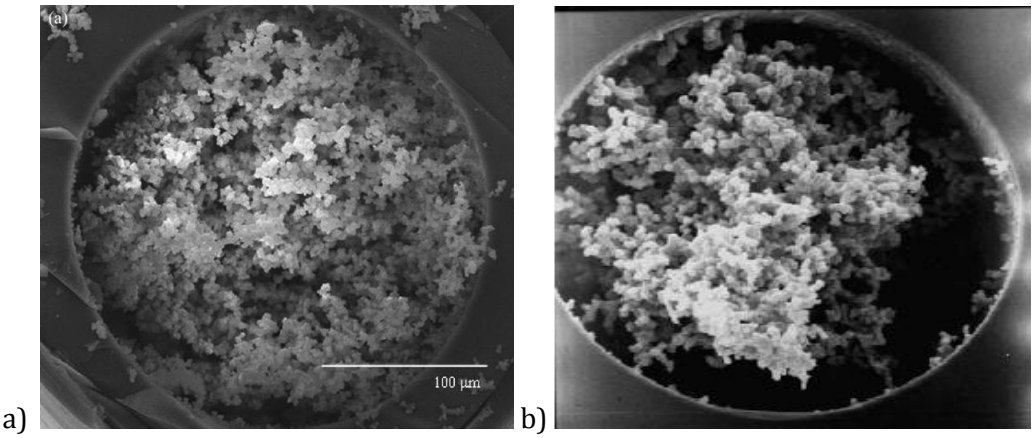


Figure 1.15: Scanning electron micrographs comparing monoliths which a) have been and b) have not been attached to the internal walls of the microfluidic channel.[70, 74]

Despite the potential of TMOS monoliths it is difficult to achieve precision placement within a microfluidic device. In light of this, research into monoliths that could be created

by photopolymerisation rather than thermal activation began. Photopolymerisation requires a photo-initiator to be added to the monomer/porogen mixture which, on exposure to ultra-violet (UV) light, facilitates polymerisation. By restricting the areas which are exposed to UV, precision placement of the monolith within the microfluidic device can be achieved.

Wen *et al*., have successfully developed a photopolymerised monolith using 3-(trimethoxysilyl)propyl methacrylate (TMSPM) as the monomer, toluene as the porogen and Irgacure 1800 as the photo-initiator, although it does require subsequent derivatisation with TMOS to achieve adequate DNA extraction efficiency.[75] Using pre-purified DNA these monoliths produced extraction efficiencies of 86%, compared with 49% for DNA extracted using a commercial Qiagen Spin Column [Qiagen, UK]. When whole blood was used as the starting material the DNA extraction efficiency of the monoliths was greatly reduced as the monolith also non-specifically adsorbed endogenous proteins present in lysed blood samples, blocking potential DNA binding sites. In order to overcome this problem, a reverse phase column, made of octadecyl (C18) coated silica beads, was included upstream of the TMSPM monolith. Proteins bind to the C18 coated beads through hydrophobic interactions which then leaves the binding sites on the TMSPM monolith free for DNA adsorption. Using this combined methodology enabled DNA extraction from whole blood with an efficiency of 69%.[76, 77]

A slight variation on the use of monoliths was presented by Chen *et al*., who used electrochemical etching of silica in the presence of an electrolyte to create a mesoporous surface.[78] Once the channel features of the microfluidic device had been etched, they were subjected to a second etching process to create a porous layer, the porosity of which could be controlled by adjusting the current, electrolyte concentration and incubation time. Pre-extracted DNA was recovered with 83% efficiency from the porous channels as opposed to 39% from the non-porous channels. While the etching process greatly

increases the surface area available for DNA adsorption, the majority of the channel is still open and so efficiency is not maximised.

1.3.5 Additional Techniques for Miniaturised DNA Extraction

The anionic nature of DNA can be exploited to enable anion exchange to be used as a DNA extraction technique. Nakagawa *et al*., coated the internal surfaces of a serpentine channel with amine groups which bind the DNA, through electrostatic interactions, at pH 7.5. Once the DNA had been washed to remove potential contaminants, the DNA was eluted by increasing the buffer alkalinity to pH 10.6 which changes the channel surface charge from cationic to neutral, thus releasing the DNA. Although DNA extraction efficiencies of 27-40% were reported, the eluted DNA is not directly compatible with downstream applications such as PCR as it is in a high salt environment.[79] By using chitosan, a natural polysaccharide produced from the partial deacetylation of chitin which is found in crab shells, as the surface coating the same principle of anion exchange can be applied but with DNA binding at pH 5 and release at pH 9 which is more suitable for downstream applications. The use of chitosan also resulted in higher DNA extraction efficiencies of 63% for genomic DNA, which may be due to the hydrophilic nature of chitosan reducing the hydrophobic interaction of proteins therefore maintaining more sites for DNA binding.[80] To further reduce the pH at which the DNA is eluted, Lien *et al*., demonstrated to the use of surface charge switchable magnetic beads for DNA extraction from white blood cells. Below pH 6 the beads carry a positive charge which can be used to capture the DNA whereas above pH 8.5 they are negatively charged and so the DNA is released.[81]

Witek *et al*., have used polycarbonate microfluidic devices in which the channels have been activated using UV radiation to produce surface carboxylate groups. Immobilisation of DNA onto the surface is achieved using a buffer containing PEG, sodium chloride and ethanol; the sample is then washed with ethanol before the DNA is eluted in a low ionic

strength buffer. While the extraction efficiency of such systems is high (85 ± 5%) the channels are dried in between the wash and elution steps which creates problems integrating this methodology with downstream applications.[82]

Kim *et al*., have used nanoporous aluminium oxide membranes to extract genomic DNA from whole blood. Following cell lysis, DNA can be separated from other blood constituents by size as it cannot pass through the pores in the aluminium oxide membrane as it is a relatively large macromolecule.[83] In addition it is hypothesised that by increasing the salt concentration of the DNA-containing solution, the negative charges on the DNA are neutralised which favours aggregation of the DNA molecules increasing adsorption on to the membrane.

All of the DNA extraction methods so far have focused on obtaining total genomic DNA, but there are a number of papers which have demonstrated sequence-specific extraction. Magnetic particles can be modified with oligonucleotide probes, for example through the use of avidin-biotin binding (Figure 1.16) [84] or phosphorous dendrimers.[85] By optimising individual reaction conditions the complementary DNA sequence can be extracted through hybridisation to the oligonucleotide probe. Archer *et al*., have shown the potential for this technology by selectively extracting adenovirus serotype 4 DNA from a sample of human DNA.[85]

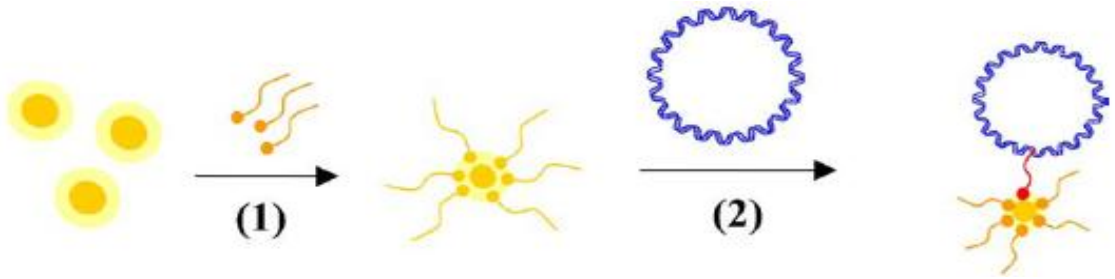


Figure 1.16: Schematic depicting sequence-specific DNA extraction. 1) Biotinylated oligonucleotide probes ( ) bind to avidin-coated magnetic particles ( ); 2) Bacterial circular genomic DNA ( ), once thermally denatured, is captured through hybridization with the oligonucleotide probe.[84]

An alternative to capturing DNA on a solid support is to carry out DNA purification by capturing the other cellular components while the DNA remains free in solution. *E. coli* cells were laser irradiated in the presence of carboxylic acid-terminated polystyrene magnetic beads causing cell lysis by heat and mechanical shock. It was shown that the released cellular proteins bound to the beads and could be drawn away from the DNA in solution using an external magnet.[23]

Prinz *et al*., showed osmotic lysis of *E.coli* cells using hydrodynamic flow followed by dielectrophoretic trapping of the released chromatin.[86] Here the entire genomic content of the cell is trapped between two electrodes while the other cellular constituents are removed (Figure 1.17). Using a similar technique, Park and Swerdlow described a method whereby crude DNA samples are concentrated and purified in a flowing stream prior to capillary electrophoresis.[87] The negatively charged DNA is captured between two gap junctions when an electric field is applied and remains in position even when hydrodynamic flow is applied. This allows interfering compounds to be removed, depending on their electrophoretic mobility, by washing using hydrodynamic flow. Elution of DNA is achieved simply by turning off or reversing the applied electric field, with recovery efficiencies of up to 95%.

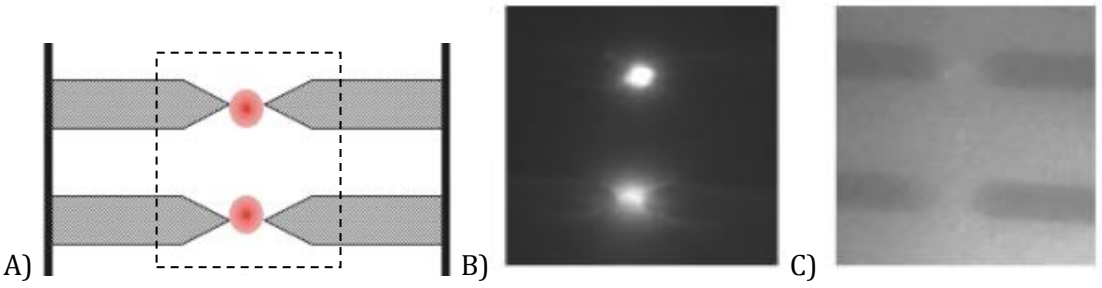


Figure 1.17: Images depicting DNA extraction using dielectrophoresis. A) Schematic showing trapping of DNA, between electrodes, using dielectrophoresis; B) Photograph showing trapping of DNA stained with an intercalating fluorescent dye; C) Photograph showing the flow through of cytoplasmic proteins, not trapped by dielectrophoresis.[86]

1.3.6 DNA Quantification

Extracted DNA can be quantified to determine the concentration of DNA within the sample. The amount of DNA used in downstream analysis is often critical, for example, PCR using the AmpFlSTR® SGMPlus™ kit optimally requires between 1 and 2.5 ng of template DNA.[12] If too little DNA is added then it is possible that some alleles may fail to amplify but if too much DNA is added then the concentration of fluorescence-labelled PCR products may be too high to permit detection within the reliable limits of sensitivity of the conventional analysis system.[2] There are a variety of techniques available for quantifying the amount of DNA present in a sample including UV spectrophotometry, fluorimetry and real-time PCR.

UV spectrophotometry can be used to monitor the absorption of DNA at 260 nm. By applying the Beer-Lambert law the concentration of DNA in the sample can be calculated.[88] This method also allows an estimate of the purity of the DNA by comparing the DNA absorbance at 260 nm to the absorbance of contaminating proteins at 280 nm. However, there are limitations as to the sensitivity of this approach and it is not possible to distinguish between different types of nucleic acids.

Intercalating fluorochromes, such as PicoGreen® and SYBR® Green, exhibit a greatly increased fluorescence when bound to dsDNA (Figure 1.18a). By comparing the intensity of the fluorescent signal obtained from a sample of unknown concentration to a series of known standards the amount of DNA present can be determined. While this type of method is specific for dsDNA it is not specific for human DNA and therefore inaccurate quantification may arise for example if there is bacterial contamination present in a sample.[2]

Real-time PCR can be used to monitor the accumulation of a specific PCR product during the amplification process from which the initial DNA template concentration can be extrapolated. This is commonly achieved using TaqMan® style fluorogenic probes, which

hybridise at a specific sequence located between the forward and reverse primers; such probes carry a fluorescent reporter dye at the 5' end and a fluorescent quencher at the 3' end (Figure 1.18bi). In this conformation the fluorescent signal from the reporter dye is quenched, but during the DNA extension step of PCR the DNA polymerase cleaves the reporter dye (Figure 1.18bii) which separates it from the quencher enabling the fluorescence to be observed (Figure 1.18biii). By using sequence-specific probes this methodology provides highly accurate quantification and eliminates the need for a subsequent separation and detection step. However, the use of specifically designed probes and expensive PCR reagents means that real-time PCR is not the most cost-effective means of DNA quantification. Whilst each of the methodologies described has its own distinct advantages and disadvantages, it is real-time PCR that has found the most applications in microfluidic systems (see Chapter 1.4.4 for details).

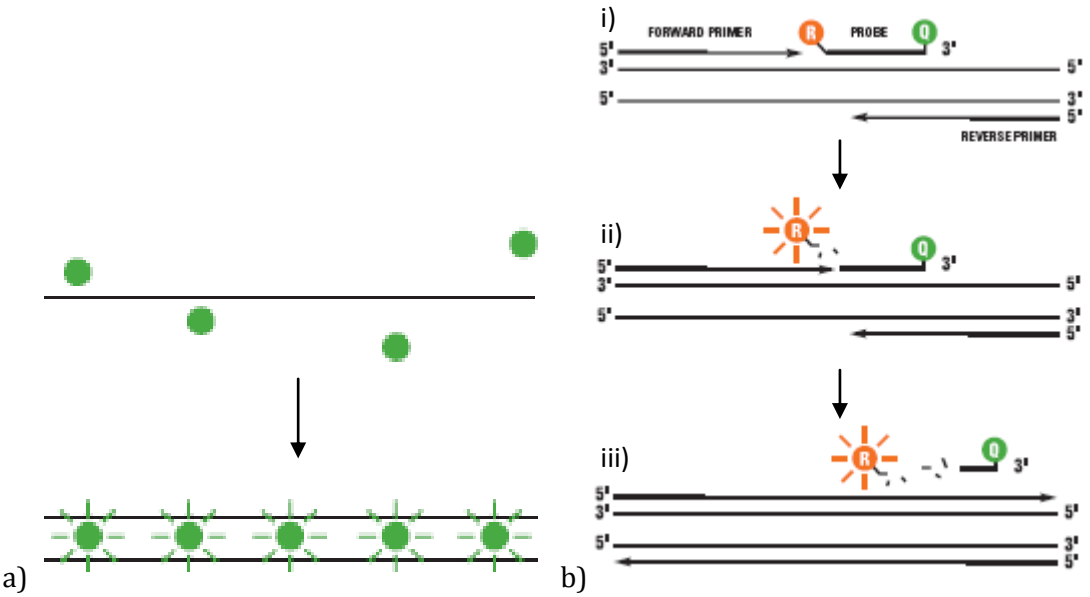


Figure 1.18: Schematic of a) Interaction of intercalating fluorochrome with ssDNA (top) and dsDNA (bottom); b) Example of real-time PCR using TaqMan® probes [Applied Biosystems, UK] where R represents the fluorescent reporter dye and Q represents the quencher. Adapted from Applied Biosystems website.[89]

1.4 Amplification of DNA Sequences

The ability to amplify template DNA present in a sample opens up the number of samples for analysis and also the number of ways in which they can be analysed. By drastically increasing the amount of DNA present, samples of low quantity and of poor quality can be investigated. Amplification aids the detection of DNA, for example fluorochromes can be incorporated into the newly synthesised DNA allowing very sensitive analysis using fluorescent-based techniques. A number of techniques for amplification of DNA exist, the most widely used being the polymerase chain reaction (PCR) which was invented by Kary Mullis and colleagues in 1985 and relies on thermal cycling through a range of critical temperatures. While PCR will be the main focus of this thesis, it is worth mentioning that in addition there are a number of isothermal techniques available. Loop-mediated isothermal amplification, nucleic acid sequence-based amplification, self-sustained sequence replication and strand displacement amplification are all examples of techniques used to perform nucleic acid amplification at a single temperature.[90]

1.4.1 Traditional PCR

PCR is a means of amplifying specific regions of DNA through a temperature dependant enzymatic reaction (Figure 1.19). Firstly, double stranded DNA is heated to 95˚C, at which temperature the hydrogen bonds holding the two strands together break and the DNA is said to be denatured. Primers are short oligonucleotide sequences (approximately 18 – 24 nucleotides) which will bind by complementary base pairing to specific sequences flanking the target sequence that requires amplification. The reaction is cooled to around 55˚C, depending on the primers in question, allowing the oligonucleotides to bind to the denatured single stranded DNA, a process known as annealing. Next the reaction is heated to 72˚C, the optimum temperature for the synthetive activity of DNA polymerase, resulting in the production of DNA strands complementary to the sequence of interest by the sequential addition of deoxyribonucleotide triphosphates (dNTPs) through a

condensation reaction. These temperatures are then cycled resulting in a theoretical exponential amplification of the DNA sequence(s) of interest.

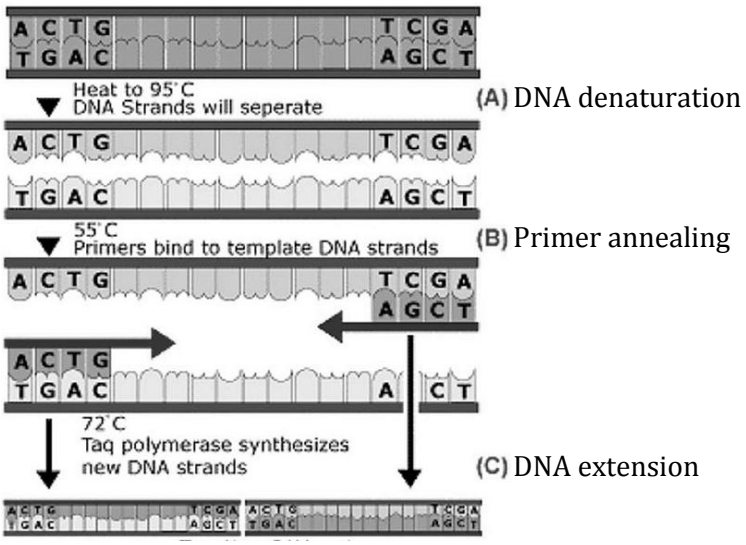


Figure 1.19: Overview of the polymerase chain reaction showing how the different temperature steps result in the amplification of DNA.[91]

PCR can be multiplexed allowing simultaneous amplification of multiple regions of the template DNA in a single reaction, achieved by including more than one set of primers in the reaction. The use of fluorescently labelled primers is beneficial during the analysis of PCR products as it enables fluorescent-based detection methods to be used. Another key component of the reaction is the DNA polymerase, which requires $MgCl_2$ as a cofactor. A wide range of DNA polymerases exist, of which the thermally stable varieties are used in PCR, as they can withstand the repeatedly high temperatures achieved during thermal cycling. The most commonly used of these is from the thermophilic bacteria *Thermus aquaticus* known as *Taq* DNA polymerase. More recently modified versions of *Taq* DNA polymerase have become available such as Hot-Start *Taq* DNA polymerases which have a chemically modified active site which renders it inactive until an initial heat denaturation step is carried out. This prevents non-specific amplification of DNA during the preparation of the reaction.[2]

Optimisation of PCR for the amplification of DNA from biological samples used in forensic analysis, requires the removal of potential inhibitors of PCR which act by preventing correct functioning of the DNA polymerase. Examples of these include proteins such as haemoglobin, inorganic ions and anticoagulants such as heparin.[82] Blood is a good example of a biological substance that contains many such inhibitors, which are commonly removed during the DNA extraction process.[73] However, more recently direct amplification from whole and dried blood samples has been demonstrated. By pre-treating samples at 95°C for 15 minutes, STR and SNP genotypic analysis could be carried out without the need to perform DNA extraction.[92, 93] The amplification process can also be assisted by diluting the blood thereby reducing the level of potential inhibitors, however this also reduces the DNA concentration. Alternatively a more inhibitor-tolerant DNA polymerase, such as T_{th} [Promega, UK], can be used or formamide can be added to the reaction which reduces protein coagulation and the melting temperature of DNA.[94] However, due to the potentially limited samples available in forensic cases, DNA extraction is essential for pre-concentration of the DNA in these instances.

PCR products are most commonly analysed either in real-time (see Chapter 1.4.3) or at the end-point using electrophoresis, by either slab-gel or capillary methodology. In both cases of electrophoresis, the PCR products are separated out based on their relative size, as the length of the product is proportional to its electrophoretic migration in the presence of an electric field whilst in a gel matrix. PCR products can either be detected using intercalating dyes, such as ethidium bromide, or by the incorporation of fluorescently labelled primers. Capillary electrophoresis is more commonly used for the analysis of PCR products from commercially available DNA profiling kits as only minute sample quantities are required and the higher voltages, permitted by improved heat dissipation, enable one base pair resolution to be achieved in minutes. The inclusion of a DNA size ladder during electrophoresis enables accurate sizing of the PCR products.[2]

1.4.2 Miniaturised PCR Methodologies

The application of PCR methodology in a microfluidic setting has shown great success due to the compatibility with miniaturisation. In particular the speed of analysis can be greatly improved due to a reduced thermal mass and the cost of analysis can be reduced due to the smaller volumes of reagents, such as DNA polymerase, required. There is a vast wealth of literature available on microfluidic PCR devices, including substantial reviews by Auroux and Zhang.[15, 95] While not every single miniaturised PCR device can be covered in this introduction a summary of the available literature is reported (Table 1.2) along with a more in depth analysis of some of the main issues regarding miniaturised PCR.

There are three main categories of microfluidic PCR devices; either stationary, flow-through or bi-directional depending on the geometry of the DNA amplification chamber, each with their own unique characteristics and inherent advantages and disadvantages. Stationary PCR involves the use of either a single chamber or an array of individual chambers in which the PCR reagents are housed and cycled through the required temperatures. The use of a stationary PCR chamber enables good fluidic and temperature control which is important in producing an optimised DNA amplification protocol. While the use of a single stationary chamber is not amenable to high throughput sampling, the development of stationary arrays for multiple parallel PCR goes some way to address this problem. The major disadvantage of performing stationary PCR is that the speed of the reaction is limited by the ramping rate of the heater.

Design	Material	Heating method	Flow Control	Volume	Passivation
Stationary chamber [96]	Glass	Block heater	Convection	35 µl	-
Stationary chamber [97-99]	Glass	Infra-Red	Pipette	270 nl - 5 µl	Silanisation
Stationary chamber [100]	Oil	Block heater	Magnetic beads	3 µl droplet	BSA
Stationary chamber‡ [101]	Oil	Thin film heater	Pipette	100 nl droplet	Fluorination
Stationary chamber [102]	PDMS	Block heater	Pipette	2 µl	Parylene
Stationary chamber‡ [103]	PDMS	Thin film heater	Pipette	0.9 µl	-
Stationary chamber [104]	PDMS - glass	Block heater	Pipette	1.75 µl	Polyethylene & BSA
Stationary chamber [17]	PMMA	Thin film heater	Pipette	200 nl	Silicon dioxide
Stationary chamber [105]	Polyimide	Infra-Red	Pipette	1.7 µl	Polyimide
Stationary chamber [106]	Polypropylene	Thin film heater	Convection	75 µl	-
Stationary chamber‡ [107]	Quartz capillary	Rapidcycler air oven	Capillary action	10 nl	BSA
Stationary chamber [108]	Silicon	Halogen lamp	Pipette	7 µl	SiO_2
Stationary chamber* [109]	Silicon	Infra-Red	Convection	20 µl	-
Stationary chamber*‡ [110-114]	Silicon	Thin film heater	Pipette	25 µl	Polypropylene inserts
Stationary chamber [115-118]	Silicon	Thin film heater	Pipette	1 - 8 µl	Silanisation or SiO_2 (& BSA)
Stationary chamber [119, 120]	Silicon-glass	Block heater	Pipette	10 µl	SiO_2 or silanisation
Stationary chamber [121, 122]	Silicon-glass	Thin film heater	Pipette	3.6 - 25 µl	BSA
Stationary chamber [123]	Silicon-pyrex	Block heater	Pipette	50 µl	-
Stationary chamber [124]	SU-8	Thin film heater	Hydrodynamic	20 µl	Silanisation
Stationary chamber [125]	Teflon capillary	Block heater	Syringe pump	24 µl	-
Stationary chamber‡ [126]	Teflon capillary	Thin film heater	Syringe pump	11 µl	-
Stationary array [127]	Glass	Block heater	Syringe pump	Variable	EGC
Stationary array‡ [128]	PDMS	Block heater	Syringe pump	25 nl	-
Stationary array* [129]	PDMS	Thin film heater	Pipette	10 µl	-
Stationary array‡ [130]	PDMS - glass	Block heater	Syringe pump	450 pl	-
Stationary array‡ [131, 132]	PDMS - glass	Thin film heater	Pipette	6 - 10 µl	-
Stationary array‡ [133-136]	Silicon	Block heater	Pipette	86 pl - 5 µl	SiO_2 (& BSA)
Stationary array [137, 138]	Silicon	Block heater	Pipette	2 - 20 µl	Polypropylene or Silanisation & BSA
Stationary array [139]	Silicon - glass	Thin film heater	Pipette	2.5 µl	SiO_2 & BSA
Flow through & Stationary‡ [140]	Quartz	Thin film heater	Vacuum pump	Droplet	Tween-20
Flow through & Stationary* [141]	Silicon	Block heater	Syringe pump	10 pl droplet	Silanisation
Flow through - spiral [142]	Polycarbonate	Thin film heater	Syringe pump	Continuous	BSA
Flow through - spiral [143]	Polycarbonate	Block heater	Syringe pump	Continuous	BSA

Flow through - serpentine [144]	Ceramic	Block heater	Peristaltic pump	Continuous	Additional Taq
Flow through - serpentine [145, 146]	Glass	Block heater	Syringe pump	Continuous	Silanisation
Flow through - serpentine* [147, 148]	Glass	Thin film heater	Syringe pump	Continuous	BSA
Flow through - serpentine [149-151]	PDMS - glass	Thin film heater	Syringe pump	Continuous	Silanisation, BSA, PEO or Tween-20
Flow through - serpentine‡ [152]	PDMS - glass	Block heater	Syringe pump	65 pl droplet	
Flow through - serpentine [153]	Polycarbonate	Block heater	Pipette	Continuous	BSA & PEG 8000
Flow through - serpentine [154]	Quartz	Thin film heater	Syringe pump	Continuous	Silanisation & Tween-20
Flow through - serpentine [155]	Silicon-glass	Thin film heater	Syringe pump	Continuous	Silanisation & BSA
Flow through - serpentine [156]	SU-8	Thin film heater	Syringe pump	Continuous	-
Flow through - helical [157]	PFA capillary	Block heater	Syringe pump	2 µl droplet	-
Flow through - helical [158]	Silica capillary	Block heater	Syringe pump	Continuous	Silanisation
Flow through - helical* [159]	Teflon capillary	Block heater	Syringe pump	Continuous	-
Flow through - helical [160]	Teflon capillary	Oil baths	Syringe pump	Continuous	-
Flow through - helical [161]	Teflon capillary	Water bath	Pressurised helium	300 nl droplet	-
Flow through - circular [162]	PDMS	Thin film heater	Compressed air	Continuous	-
Flow through - circular‡ [163]	PDMS	Thin film heater	Peristaltic pump	Continuous	-
Flow through - circular [164]	PMMA	Block heater	Ferrofluid	Continuous	-
Flow through - circular [165]	Polycarbonate	Block heater	Electrokinetic	Continuous	Polybrene
Flow through - circular [166]	Teflon capillary	Block heater	Convection	Continuous	-
Bi-directional‡ [167]	Glass	Block heater	Syringe pump	2 µl	Silanisation, PVP, BSA & Tween-20
Bi-directional* [168]	PDMS	Block heater	Pneumatic pump	100 nl	poly(L-lysine) & PEG
Bi-directional‡ [169]	PDMS – glass	Joule heating	Electrokinetic	0.4 µl	-
Bi-directional [170]	PTFE capillary	Block heater	Pressure driven	1 µl	-

Table 1.2: Summary of the properties of different types of microfluidic DNA amplification devices reported in the literature (N.B. This is not a fully comprehensive list). Devices capable of performing more than one technique in an integrated device, e.g. PCR and CE, have not been included as they will be discussed in more detail in Chapter 1.5. Those devices capable of performing real-time detection are indicated by * for intercalating dye and ‡ for TaqMan™ probes.

In a flow-through device the PCR reagents are continuously passed through distinct temperature zones resulting in thermal cycling. A number of flow-through designs have been investigated, namely serpentine, circular and bi-directional (Figure 1.20).

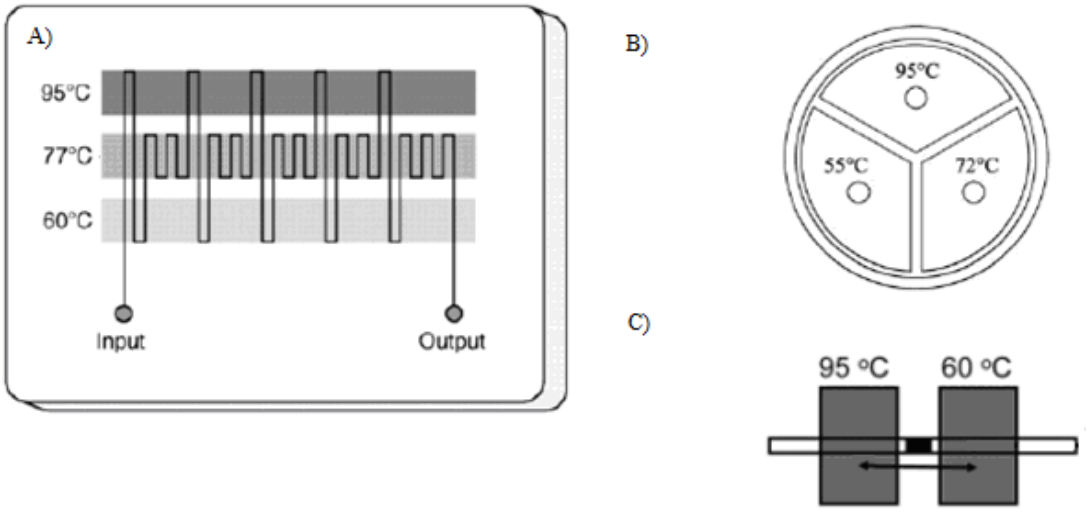
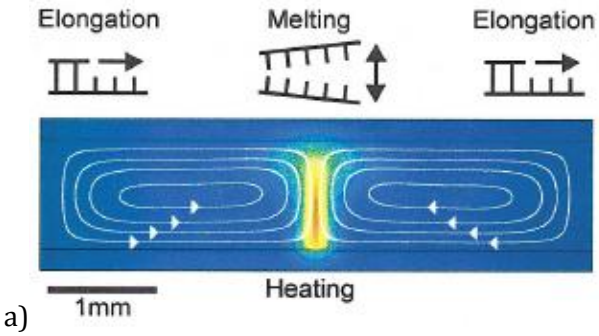


Figure 1.20: Designs for flow-through DNA amplification devices A) serpentine,[145] B) circular [159] and C) bi-directional flow.[167]

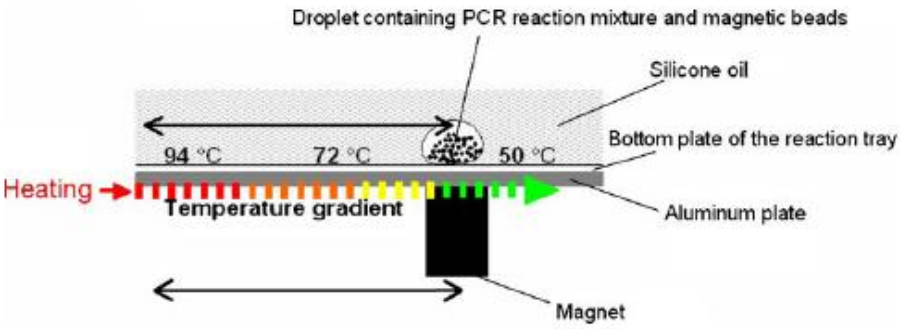
The reliance on discrete temperature zones gives rise to rapid heat transfer which in turn can result in faster cycling times, depending upon the flow rate of solutions through the microfluidic device. High sample throughput is also achievable using flow-through systems, either by continuous PCR of one type of sample or analysis of multiple samples in droplet form. The main disadvantage of serpentine flow-through PCR is that the cycle number is fixed by the geometry of the microfluidic device which reduces the flexibility of the system, although recent developments have looked at the use of bi-directional flow-through PCR in which the cycle number is not limited. The use of bi-directional flow-through, however, tends to be limited to two-step DNA amplification reactions due to the location of the heating elements at either end of the bi-directional flow.

Recently, the use of droplet-based PCR has emerged as a rapidly expanding field for high throughput DNA amplification. In such systems, water in oil droplets containing all the necessary PCR reagents are applied to flow-through devices. The confinement of PCR reagents to droplets confers a number of advantages over conventional flow-through PCR systems including: elimination of sample-to-sample contamination, reduced DNA polymerase adsorption to the internal surfaces of the microfluidic device and a reduction in local temperature variation.[171] Perhaps most importantly, the use of droplet-based PCR enable the encapsulation and subsequent amplification of single molecules of DNA, allowing single cell analysis to be performed.[172]

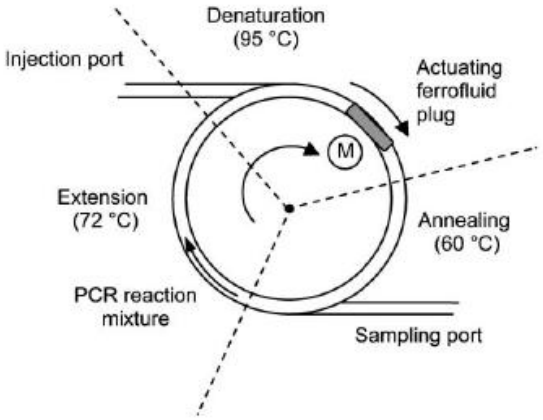
In order to achieve movement within a flow-through system a wide range of techniques have been examined; the most common of these is the use of hydrodynamic pumping achieved through the use of syringe or peristaltic pumps. While it is possible to generate highly controlled flow rates, there are difficulties in integrating external pumps whilst maintaining a miniaturised footprint for the microfluidic system. A number of alternative systems have therefore been investigated including the exploitation of convection currents to achieve movement of solutions.[96, 106, 109, 166] By maintaining two heating elements at the temperatures required for denaturation and annealing/extension naturally occurring Rayleigh-Bernárd convection forces the circular movement of solutions around the chamber (Figure 1.21a). Magnetic forces have also been investigated for movement of PCR reagents during thermal cycling in flow-through microfluidic devices. Ohasi *et al*., added magnetic beads to aqueous droplets of PCR reagents in an oil carrier and used an external magnetic to repeatedly move the beads along a thermal gradient (Figure 1.21b).[100] Whereas Sun *et al.,* propelled PCR reagents through the different temperature zones in a closed circular device using a ferrofluidic plug driven by the rotation of an external magnet (Figure 1.21c).[164] Electrokinetic pumping has also been shown to be successful for the manipulation of PCR reagents in a microfluidic device, with Hu *et al.,* even demonstrating that the Joule heating created by electrokinetic movement could in addition be used to thermally cycle the PCR reagents (Figure 1.21d).[165, 169]



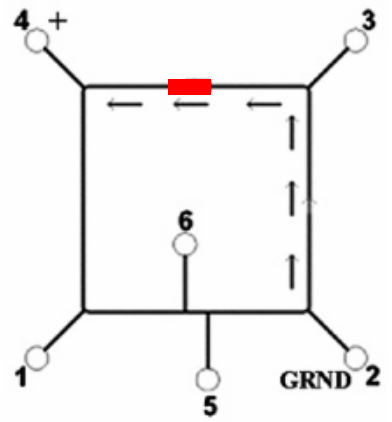
a)



b)



c)



d)

Figure 1.21: Examples of flow control methods within microfluidic PCR devices: a) Rayleigh-Bernárd convection;[109] b) manipulation of a PCR reagent droplet, containing magnetic beads, along a temperature gradient;[100] c) PCR reagents driven through different temperature zones using a ferrofluid plug controlled by an external magnet (M);[164] d) electrokinetically-driven movement of PCR reagents (▬) around a continuous flow device, e.g. movement up the right arm and along the top arm by applying an electric field between electrodes at positions 2 and 4 .[165]

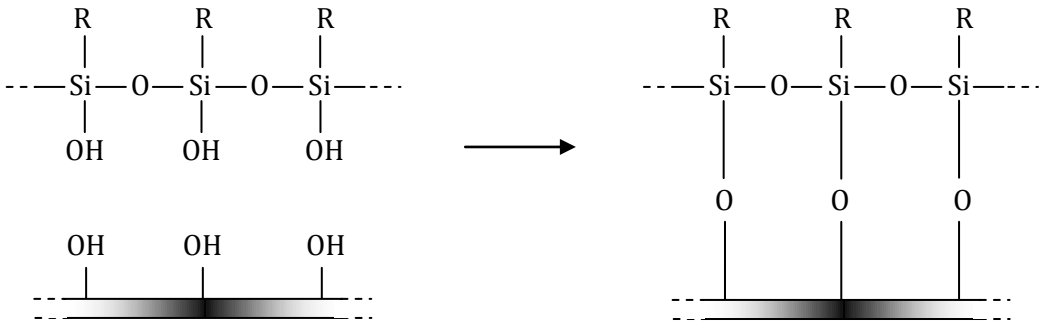
In a conventional benchtop thermal cycler the average PCR volume is ~50 µl, miniaturisation drastically reduces the volume of PCR reagents required which in turn reduced the cost of the reaction. Successful PCR in picoliter size volumes on microfluidic devices have been reported, representing around a 10^6 fold decrease in sample volume required and hence a significant cost saving.[135, 141] While it has been demonstrated that PCR can be performed on such as small scale, there are still major hurdles in overcoming the so-called 'world-to-chip' interface i.e. converting from the macro to the micro scale where there is a minimum practical volume of ~1µL which can be injected into a system. In answer to this a number of approaches have been developed, for example, droplet generation enables picoliter size volumes of PCR reagents to be produced on a microfluidic device based on their immiscibility in an oil carrier phase.[141, 152, 157, 161] For performing stationary PCR on a small scale, Liu *et al.,* created a matrix array of 400 3nl PCR reactors which, through series of valves and pumps, just 2 µl of DNA polymerase could be equally distributed.[173]

A wide variety of methods are available for heating and cooling of PCR reagents to achieve thermal cycling, as indicated in Table 1.2. Contact heating methods include the use of thin film and block heating elements, such as Peltier heaters, which have the advantages of fit-for-purpose size and precision temperature control. However, they can suffer from large thermal mass, slow temperature ramp rates and non-transparency. Non-contact heating methods include the use of hot air, infra-red and halogen lamps each with their own distinct advantages and disadvantages. One interesting example is the use of microwaves to provide thermal cycling, not on a microfluidic scale, but to dramatically increase the volume of PCR sample which can be analysed enabling increased sample processing. Orrling *et al.,* presented a microwave heating system capable of performing PCR in a 15 ml reaction volume with controlled temperature uniformity over the entire sample volume.[174]

The high surface area to volume ratio present in microfluidic systems can create unique problems. Miniaturised PCR is a perfect example of this as it has been demonstrated that

at the high surface area to volume ratio present, DNA polymerase adsorption occurs resulting in inhibition of the DNA amplification reaction.[122] Prakash *et al,* investigated this phenomenon further and were able to categorise microfluidic device materials into two types: propagating adsorption materials and contained adsorption materials. The former exhibits progressive adsorption of the DNA polymerase e.g. SU-8, whereas the latter shows an initial instantaneous adsorption but then no further adsorption takes place e.g. Teflon.[175] As most microfluidic devices are manufactured from materials which exhibit progressive adsorption, surface passivation techniques have been developed to prevent loss of DNA polymerase to the surface which can broadly be divided into two categories: static or dynamic.

Static passivation is where the internal surfaces of the device are treated to prevent interaction with the reagents. Coating of the internal surfaces through the use of Surfasil™ or epoxy-(poly)dimethylacrylamide (EPDMA) has been shown to increase the reproducibility and yield of the PCR compared to untreated surfaces.[176-178] However, the use of silanisation techniques has been shown to be more effective, yielding 81% product compared to 72% for EPDMA.[177, 178] Silanisation is the process whereby free silanol groups on the surface of a material react with the silanising reagent groups.



(Equation 1.4)

Silanisation can be achieved using commercially available products such as SigmaCote™ [Sigma-Aldrich, UK] or SafetyCoat™ [JT Baker, US]. Silanisation coatings can be removed

using sodium hydroxide allowing reusing of microchips without contamination.[179] Other successfully applied static passivation techniques include the deposition of oxide layers[17, 108, 139, 176] or the application of the Hjertén coating, which involves polymerisation of linear poly(acrylamide) onto glass via a covalently bonded silane linker.[180]

Chen *et al.* demonstrated that silanisation alone was not sufficient to prevent DNA polymerase adsorption and that dynamic passivation was required as well, whereby additional reagents are added to the solution to limit the interaction of DNA polymerase with the surface.[167, 181] One of the simplest solutions is to increase the DNA polymerase concentration above a point at which the internal surface of the microfluidic device becomes saturated but this is a costly measure. A more cost-effective treatment is to include bovine serum albumin (BSA), a protein which is adsorbed competitively onto the surface thereby limiting polymerase adsorption, into the mixture.[167] Other additives which can be used to reduce DNA polymerase adsorption include poly(ethylene glycol) (PEG),[153] poly(ethylene oxide),[182] poly(vinylpyrrolidone) (PVP),[167] poly(L-lysine) [168] or Tween-20.[154] However, it should be noted that some DNA polymerase will still absorb on the microdevice surface as a result of dynamic equilibrium between reagents which is why dynamic passivation is usually carried out in conjunction with a static method.[122, 183]

1.4.3 Miniaturised Real-Time PCR

The ability to monitor the accumulation of DNA amplification products in real-time has several advantages over conventional end-point PCR. It allows accurate quantitation of the initial template DNA concentration during the exponential phase of the reaction and prevents the need for post-PCR product analysis. As indicated in Table 1.2, a number of miniaturised PCR devices have also incorporated the ability to monitor the accumulation of PCR products, using either intercalating dye or TaqMan™ probes. Both techniques rely on the increase in fluorescence intensity as the amount of amplified DNA increases

(the mechanisms are as described in Chapter 1.3.6 on DNA quantification). Fluorescence measurements are taken every cycle and the relative intensity plotted against cycle number giving rise to a sigmoidal curve if PCR products are detected (Figure 1.22).

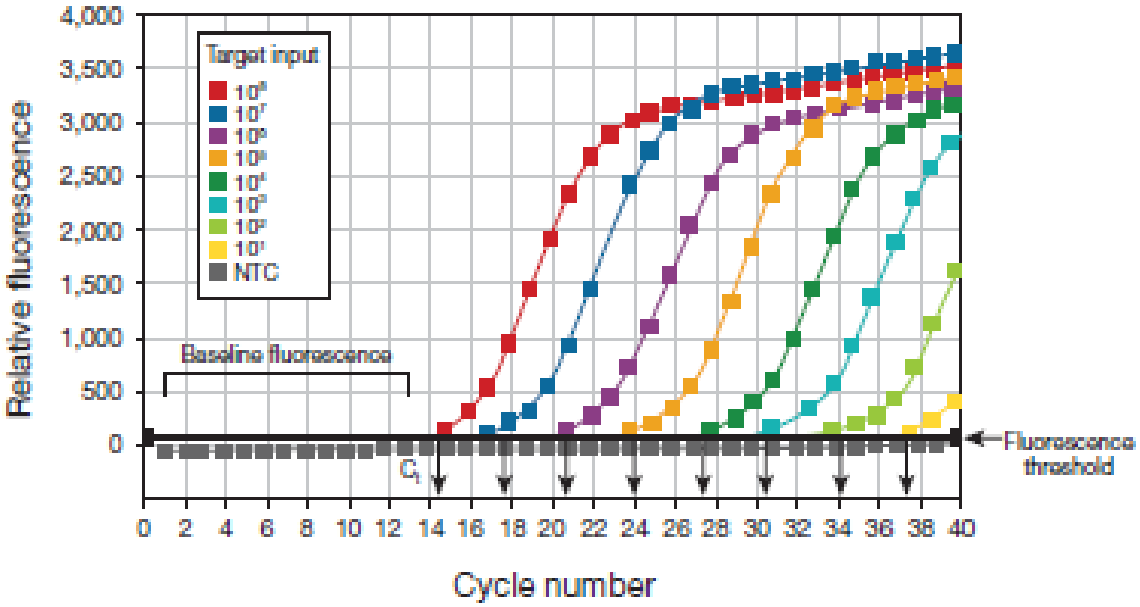


Figure 1.22: Example plot of real-time PCR analysis showing the effects of initial DNA template concentration on C_T value.[184]

The greater the amount of initial template DNA the sooner the fluorescent signal will rise above the baseline; the cycle number at which the fluorescent signal reaches a defined threshold (C_T) can be compared to a series of DNA standards of known concentration enabling accurate quantitation. Real-time PCR has been successfully applied to both stationary, flow-through and bi-directional microfluidic devices (see Table 1.2).

1.5 Integrated Microfluidic Devices for Genetic Analysis

The move towards the production of portable systems capable of genetic analysis has led to the integration of multiple processes on single microfluidic devices. Publication of papers that report integrated microfluidic systems has followed closely the publication of single process microfluidic devices (Figure 1.23). In 1993 the first miniaturised PCR device was described by Northrup and colleagues, who built a stationary PCR chamber in a silicon microdevice.[185] Three years later the first device which combined PCR with capillary electrophoresis was developed by Woolley *et al.*[186] DNA profiling requires DNA extraction and amplification to be coupled to electrophoretic separation and fluorescence based detection of the amplified loci. This requires integration of these several processes which necessitates compromise between each individual component. For example, the number of PCR cycles can be adjusted depending on the sensitivity of the detection system. While the integration on 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 (Figure 1.24).

In order to combine DNA extraction and amplification on a single microfluidic system there are a number of issues which must be taken into account including; confining the solid-phase extraction matrix, preventing contamination of the DNA amplification region with potential inhibitors from the DNA extraction process e.g. guanidine hydrochloride and isopropanol, and isolating any surface coating required for prevention of DNA polymerase adsorption to the PCR region.[72]

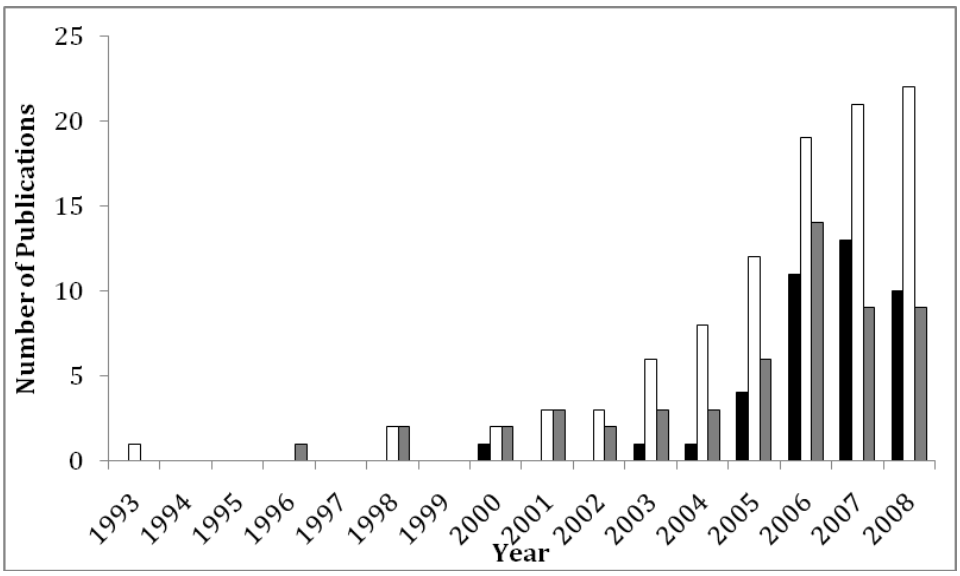


Figure 1.23: Number of microfluidic publications per year, published on Web of Science, relating to DNA extraction (■), PCR (□) or integrated systems containing either DNA extraction or PCR with at least one additional technique (■). The following search terms were used to generate the above data: "DNA extraction AND microfluid*", "DNA purification AND microfluid*", "DNA amplification AND microfluid*" or "polymerase chain reaction AND microfluid*".

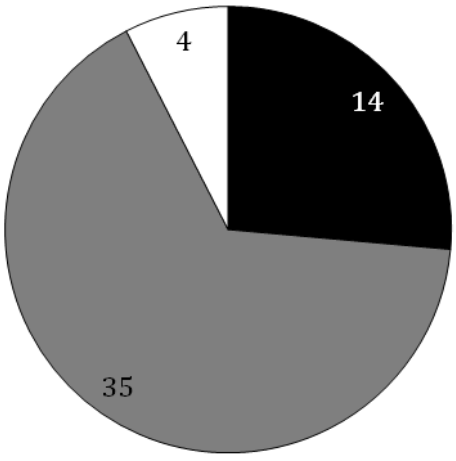


Figure 1.24: Breakdown of the number of published integrated microfluidic papers which report PCR combined with upstream processing such as DNA extraction (■), downstream processing such as capillary electrophoresis (■) or both (□).

Initial work on integrating processes upstream of PCR on microfluidic devices focused on the isolation of specific cell types from complex samples followed by thermal lysis and subsequent PCR. For example, white blood cells can be isolated from whole blood using weir-type filters which allow the smaller red blood cells to pass through. The white blood cells can then be thermally lysed and the DNA liberated directly amplified within the microfluidic device.[28, 187] Cheong *et al*., presented a device for one-step extraction and real-time PCR of pathogens in a single chamber, where gold nanoparticles were used to transform near IR energy into thermal energy facilitating cell lysis and PCR cycling.[188] Whilst some degree of DNA purification is achieved by such methods, combining cell lysis and DNA amplification means that potential endogenous inhibitors of PCR will remain.

One way to overcome the challenges of integrated DNA extraction and amplification is to use magnetic particles to capture DNA during the extraction process and then directly transfer the bound DNA into a chamber with PCR reagents, where amplification is performed in the presence of the magnetic particles.[81] An alternative use of magnetic particles was presented by Lee *et al*., where by cell lysis was performed in the presence of carboxylic acid-terminated polystyrene coated magnetic beads within a PCR reagent solution on a microfluidic device.[23] The magnetic beads bound proteins released from the cell while the DNA remained free in solution, although it was interesting to note that no binding of the DNA polymerase occurred. The magnetic beads, along with potential inhibitors, were then drawn to the side of the amplification chamber using a magnet and PCR performed and monitored in real-time. However, it has been demonstrated that by performing PCR in the presence of magnetic beads the amplification efficiency can be decreased by as much as 50%.[189]

An alternative approach is to combine the flow of DNA eluted from a silica-based solid-phase extraction matrix with a concentrated PCR reagent mixture from a side channel and direct the combined solution into a PCR chamber for direct amplification.

For example, Cady *et al*., eluted DNA from microfabricated silica pillars and combined this with a PCR mixture containing SYBR Green for real-time fluorescent-based detection.[190] Landers' group has shown that DNA can be eluted from silica bead/ sol-gel matrices and combined with PCR reagents in the same manner prior to IR-mediated thermal cycling.[191, 192] While this method has proved successful it relies on the use of a concentrated PCR reagent mix which, due to the expense of DNA polymerase enzymes in particular, seems counterintuitive in a microfluidic system where one of the main advantages is reduced reagent consumption and therefore cost of analysis.

Despite recent developments in the production of integrated microfluidic devices for performing multiple processes for genetic analysis, there still remain a number of challenges achieving truly portable, integrated DNA analysis systems. Some of the most complete systems reported for integrated genetic analysis have been reported by the Landers group at the University of Virigina and the Mathies group at the Univeristy of California, Berkeley.

In 2006, Easley *et al*., reported a completely integrated genetic analysis device with sample-in-answer-out capability for detection of *Bacillus anthracis* from whole blood samples which represented a major advancement in miniaturised genetic analysis.[192] A four layer glass-PDMS microfluidic device was used in which flow control was achieved using a single syringe pump in combination with five elastomeric valves. The whole blood sample was mixed with lysis buffer and then manually injected on to the microfluidic device. DNA extraction was performed using silica beads and the eluted DNA stream mixed with a concentrated PCR reagent solution stream *en route* to the DNA amplification chamber. Thermal cycling of the 550 nl PCR chamber was provided using IR heating and compressed air, providing heating and cooling rates of 10°C/second and 20°C/second respectively. Following DNA amplification, PCR product was pressure injected onto the separation domain along with a DNA size standard. Electrophoretic separation and laser

induced fluorescence were used to detect the PCR product which was labelled using an intercalating dye. The total analysis time, from sample input to production of the electropherogram, was less than 25 minutes.

More recently, Liu *et al*., carried out forensic analysis at a mock crime scene using an integrated microfluidic system capable of producing a CODIS hit within 6 hours of sample collection.[193] Similarly to the work by Easley *et al.,* a four layer glass-PDMS microfluidic device was used which contained elastomeric valves controlled by hydrodynamic pumping. In addition, components for thermal regulation were also fabricated as integral parts of the microfluidic device. The mock crime comprised blood samples from both suspect and victims which were subjected to analysis using the integrated microfluidic system. DNA extraction and pre-concentration was performed 'off-chip' using commercial kits. The DNA sample was then manually injected into a 160 nl PCR chamber along with all the necessary reagents to perform amplification using a custom-made multiplex consisting of eight STR loci and the Amelogenin sex marker. Contact heating methodology was employed which enabled 32 cycles to be performed in two hours. Following DNA amplification the PCR products were co-injected with a DNA size ladder for capillary electrophoresis. Laser induced fluorescence was used to detect the separated PCR products resulting in the generation of a DNA profile. Differences between the published literature described above and the work presented within this thesis are discussed in Chapter 6.

Despite such advances by both the Landers and Mathies groups, an explosion in published literature or commercialisation of such integrated systems has not been forthcoming. Microfluidic devices capable of performing integrated analysis often have complex design features e.g. microvalves, multiple layers/substrates, which are difficult to fabricate. This limits the ability to mass produce low-cost, disposable systems which are essential for performing genetic analysis in a cost-effective manner whilst ensuring accurate results by limiting the possibility of contamination.

Mechanisms for simplifiying the integration of different processes on a single microfluidic device are therefore still required.

The integration of multiple processes on a single microfluidic device also requires an operating system capable of controlling all the external components necessary to achieve sample processing. External syringe pumps are frequently required to transfer reagents onto, and control movement within, the microfluidic device. In addition, other external components often include; a heating system for DNA amplification, a system for electrophoretic separation and a detection system e.g. laser and spectrometer. Such an operating system must also be miniaturised and robust enough for the development of an 'at-point-of-use' system, preferably with the capability to be battery-powered to aid portability. These challenges must be overcome in order to allow miniaturised genetic analysis systems to successfully move from the research laboratory into the real-world.

1.6 Aims

This PhD project forms part of a larger research effort to develop a portable, integrated DNA processing/analysis instrument for 'at the scene of crime' use with remote operational capability. While a wealth of literature is available on the miniaturisation of each of the individual techniques required for performing DNA analysis, it is the integration of these individual components that is a significant challenge.

For this specific application, the microfluidic device must be capable of extracting DNA from biological samples, performing DNA amplification of multiple forensically relevant loci followed by electrophoretic separation and fluorescence detection of the individual amplified allelic sequences. In order to create a portable instrument, speed of analysis and low power consumption are important factors. A schematic of the proposed instrumentation, detailing all the necessary components required for performing all the above processes for DNA analysis (Figure 1.25).

The work reported here aims to develop the initial processes required for DNA analysis, namely DNA extraction and amplification, in an integrated microfluidic environment. Specifically this will include:

- The evaluation of different solid-phase matrices for DNA extraction.
- The optimisation of fluidics and surface properties in order to optimise the extraction efficiency of DNA from a range of biological sample types
- The design of the DNA extraction and amplification chambers (including integration with the 'portable operating box' and other microfluidic device components)
- The investigation of thermal cycling using microwave and conventional resistive heating

- The evaluation of DNA amplification performance on the microfluidic device for the analysis of specific phenotypic traits and multiple STR loci

- The development of methodology for producing a ready-to-use microfluidic device, in which all the necessary reagents are pre-loaded

- The integration of DNA extraction and amplification with further downstream applications

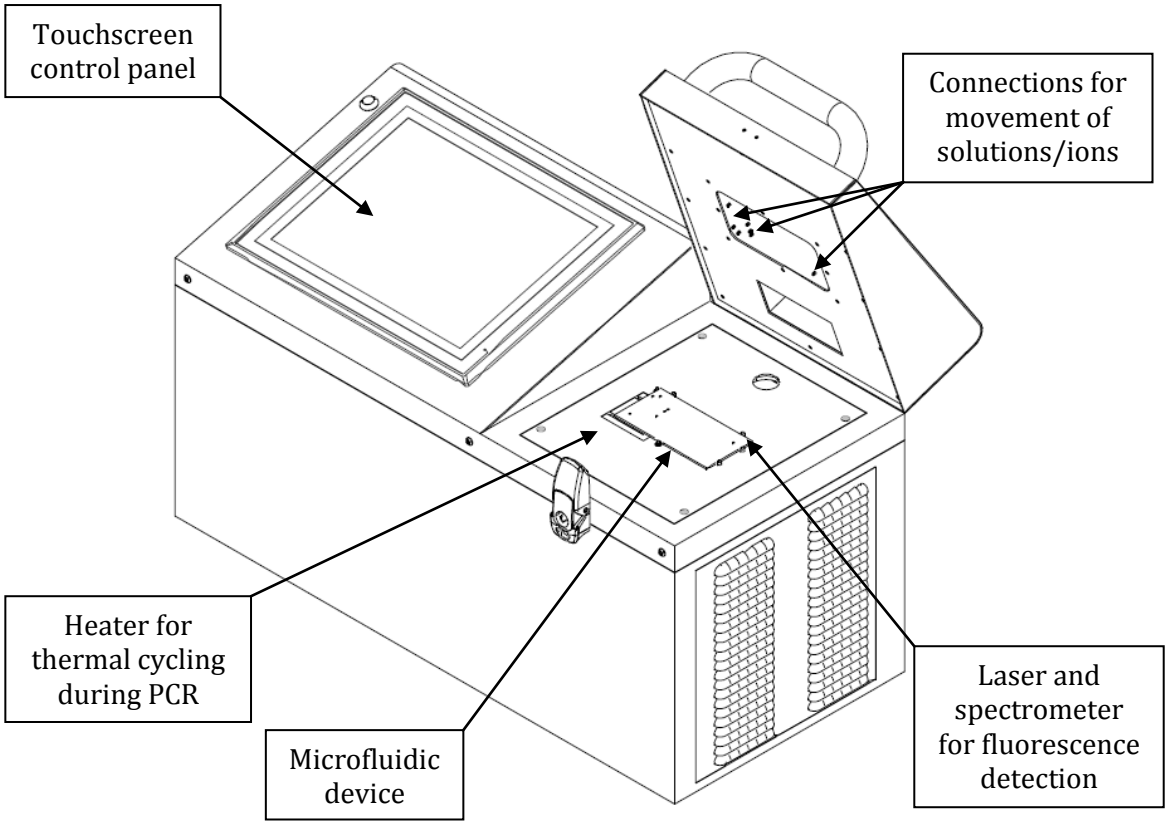


Figure 1.25: Schematic of the proposed device for performing all the integrated processes required for DNA analysis on a biological sample and produce a complete DNA profile. Once the microfluidic device has been added to the system, connectors facilitate the interface between the device and external pumping mechanisms which are capable of hydrodynamic or electrokinetic movement of reagents. A heating system is required for thermal cycling as part of the DNA amplification process. Detection of the fluorescently-labelled PCR products is achieved using a laser and a spectrometer in combination.

2 Materials & Methods

This chapter aims to provide a general overview of the materials and methods employed, along with detailed descriptions of the equipment used. More detailed methodology regarding the individual experiments carried out is given in subsequent chapters.

2.1 Fabrication of Microfluidic Devices

All glass microfluidic devices were fabricated by Dr. Steve Clark at the University of Hull using photolithography and wet etching techniques. A schematic of the processes involved is given in Figure 2.1. Masks containing design features for DNA extraction, PCR and capillary electrophoresis were drawn by Dr. Peter Docker (University of Hull) using AutoCad® and printed [JD phototools, UK]. Details of the microfluidic device designs are given in the relevant chapters, depending on their function (Figures 3.1, 4.1 and 5.1). Borosilicate glass of 1 mm thickness was used which has a vapour deposited chrome layer on one side, on top of which is a spin-coated photoresist layer [Telic, US]. The transfer of channel/cavity geometries was carried out by placing the mask over the top of the photoresist and exposing to ultraviolet (UV) light for 1 minute. After development of the photoresist layer, the exposed resist and chrome layer were removed by sequential treatment with Microposit© Developer [Chestech Ltd, UK] (diluted 50:50 with water) for 1 minute and Chrome Etch 18 Solution [Chestech Ltd, UK] for 1 minute. The exposed glass was then isotropically etched in 1% hydrofluoric acid / 5% ammonium fluoride solution at 65°C producing an etch rate of approximately 5 µm/min. Any remaining photoresist and chrome was removed using Microposit© Remover 1165 and Chrome Etch 18 Solution respectively. In order to allow the microfluidic device to be connected to hydrodynamic pumps or electrodes, 1 mm holes were drilled in the top plate. The etched top and bottom plates were then thermally bonded together at 595°C for 3 hours to produce the complete microfluidic device.

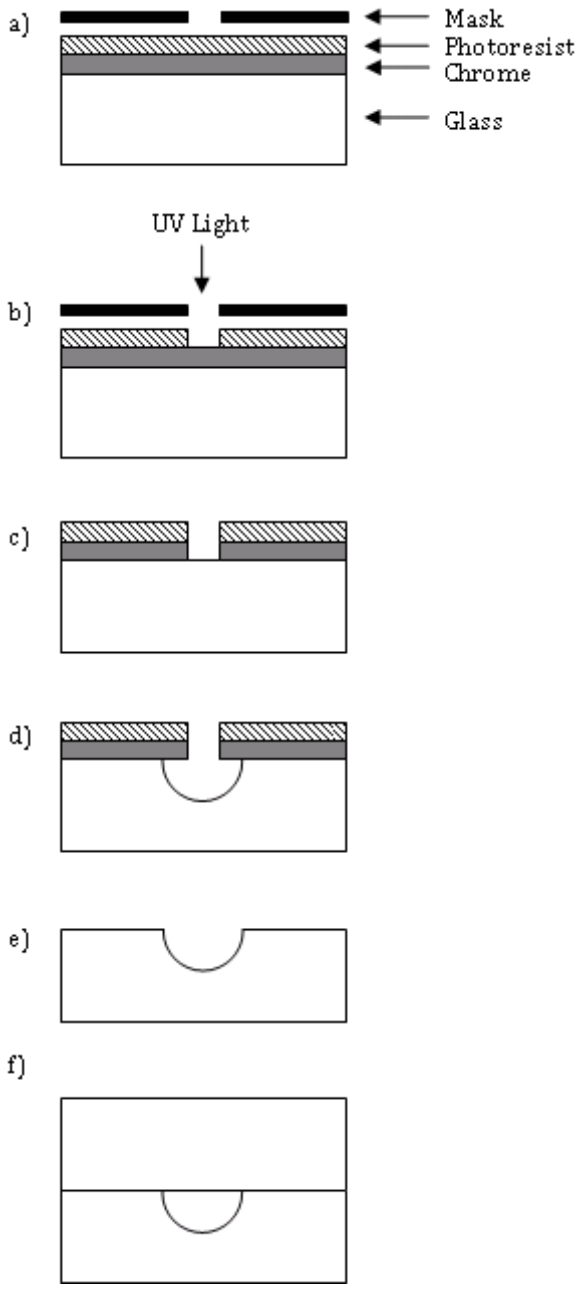


Figure 2.1: Schematic of wet-etching technique for production of glass microfluidic devices: a) mask placed on top of borosilicate glass with chrome and photoresist layers, b) UV light transfers mask design to photoresist layer, c) transfer of mask design through chrome layer, d) etching of glass surface to produce design, e) removal of remaining photoresist and chrome layers, f) completion of microfluidic device by bonding of two glass plates. Adapted from McCreedy *et al.*[194]

2.2 Biological Samples

2.2.1 Collection and Preparation of Biological Samples

A variety of biological sample types were used to simulate forensically relevant specimens, including buccal cells, saliva and whole blood. To collect buccal cells as a source of DNA an OmniSwab [Whatman, UK] was gently scraped against the inside cheek. The end of the swab was then ejected into a 1.5 ml polypropylene microcentrifuge tube and suspended in either Qiagen ATL (lysis) buffer [Qiagen, UK] or guanidine hydrochloride (GuHCl) [Sigma-Aldrich, UK] in 10 mM TE buffer solution (10 mM Tris-HCl [Sigma-Aldrich, UK] plus 1 mM ethylenediaminetetraacetic acid (EDTA) [Sigma-Aldrich, UK] in purified water, adjusted to pH 6.7 using hydrochloric acid [Sigma-Aldrich, UK]).

Saliva was collected using a 0.9 % (w/v) saline solution mouthwash, prepared by dissolving 0.9 g sodium chloride (NaCl) in 100 ml purified water and autoclaving. The saline solution was then swilled around the mouth of a volunteer and collected in a 15 ml falcon tube. A 1 ml aliquot of the mouthwash was placed in a 1.5 ml polypropylene microcentrifuge tube and centrifuged at 14,000 rpm for 3 minutes. The supernatant was removed and the pellet resuspended in either Qiagen ATL buffer or GuHCl solution.

A lancet was used to obtain a 10 µl droplet of blood from the finger of a volunteer, which was then added to a 0.2 ml polypropylene tube [Alpha Laboratories, UK]. Qiagen ATL buffer or GuHCl solution was either immediately added or the blood was left to air dry for up to 14 consecutive days prior to addition. A 1µl aliquot of Proteinase K (ProK), 10 µg/µl, was then added in some cases to aid cell lysis by heating the sample to 56°C in a water bath for 10 minutes.

2.2.2 Conventional DNA Extraction using QIAamp® DNA MicroKits

In order to evaluate the efficiency of the DNA extraction process on the microfluidic device, DNA of known quantity was required (Equation 2.1).

$$\text{DNA extraction efficiency (\%)} = \frac{\text{Amount of DNA recovered during the elution phase (ng)}}{\text{Amount of DNA initially added to the system (ng)}}$$

(Equation 2.1)

QIAamp® DNA Micro Kits [Qiagen, UK] were used to obtain purified DNA, which could be quantified for extraction efficiency studies, and also to provide a conventional technique to which the microfluidic device DNA extraction procedure could be compared. The exact composition of reagents from the QIAamp® DNA Micro Kit is unknown due to proprietary constraints so just their names and functions are given here.[46] To the biological sample, 400 µl of ATL (lysis) buffer and 20 µl ProK was added, before the solution was incubated at 56 °C for 1 hour. Next 400 µl AL (binding) buffer was added and the sample incubated at 70 °C for 10 minutes, after which 300 µl 100% ethanol was added. The lysate was transferred to a QIAamp MiniElute™ Column and washed sequentially with 500 µl AW1 and AW2 (wash) buffers. The column was then spun dry before the DNA was eluted in 50 µl purified water. Samples were stored, in 10 µl aliquots, at -20°C until required.

2.3 Quantification

2.3.1 DNA Quantification

DNA quantification was achieved using a Quant-iT™ PicoGreen® dsDNA Assay Kit [Invitrogen, UK] which contains PicoGreen®, a fluorochrome which selectively binds dsDNA. PicoGreen™ has an excitation maximum of 480 nm and emission at 520 nm.[195]

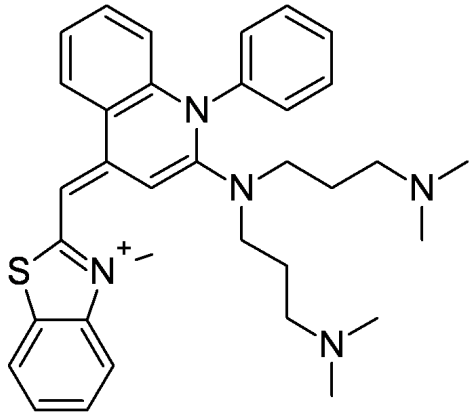


Figure 2.2: Structure of PicoGreen™.

The PicoGreen® stock solution was diluted 1 in 400 in 1x TE buffer to produce a working solution. To each 2 µl aliquots of sample from the DNA extraction process was added 50 µl of the PicoGreen® working stock solution 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. Standards were made up in either GuHCl solution, alcohol or 10 mM TE buffer, to allow a direct comparison with samples collected at each of the stages of the DNA extraction procedure. A blank (no DNA control) was also used to account for any background fluorescence. All samples were analysed using a FLUOstar Optima Plate Reader [BMG Labtech, UK]. DNA standards were ran in duplicate and a calibration curve, typical of that seen in Figure 2.3, was produced automatically using FLUOstar Optima software against which the DNA samples of unknown concentration were compared.

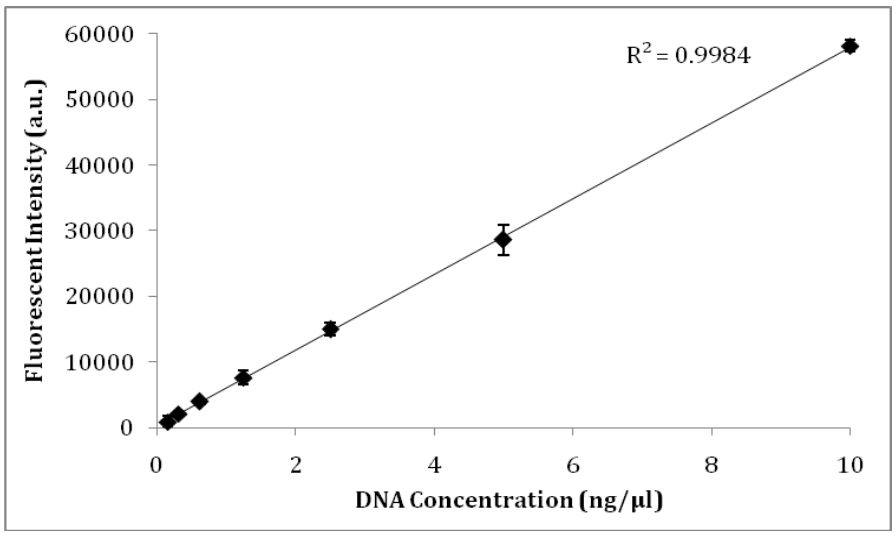


Figure 2.3: Example of a calibration curve produced for PicoGreen™ using λ–phage DNA standards, analysed using a FLUOstar Optima Plate Reader.

2.3.2 Protein Quantification

Protein quantification was achieved using a Pierce® BCA Protein Assay Kit [Thermo Scientific, UK]. The kit contains bicinchoninic acid (BCA) which enables colorimetric detection and quantitation of total protein. A working reagent solution was produced by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. To each 10 µl aliquot of sample from the DNA extraction process was added 200 µl of the working reagent solution in a clear microtitre plate. The plate was then incubated at 37°C for 30 minutes prior to the absorbance being read at 562 nm using a FLUOstar Optima Plate Reader. Protein standards, using BSA, provided a calibration curve at the following concentrations: 1000, 500, 250, 125, 62.5, 31.25 and 15.625 ng/µl. Standards were made up in either GuHCl solution, alcohol or 10 mM TE buffer, to allow a direct comparison with samples collected at each of the stages of the DNA extraction procedure. A blank (no protein control) was also used to account for any background absorbance. Protein standards were ran in duplicate and a calibration curve, typical of that seen in Figure 2.4, was produced

automatically using FLUOstar Optima software against which the protein samples of unknown concentration were compared.

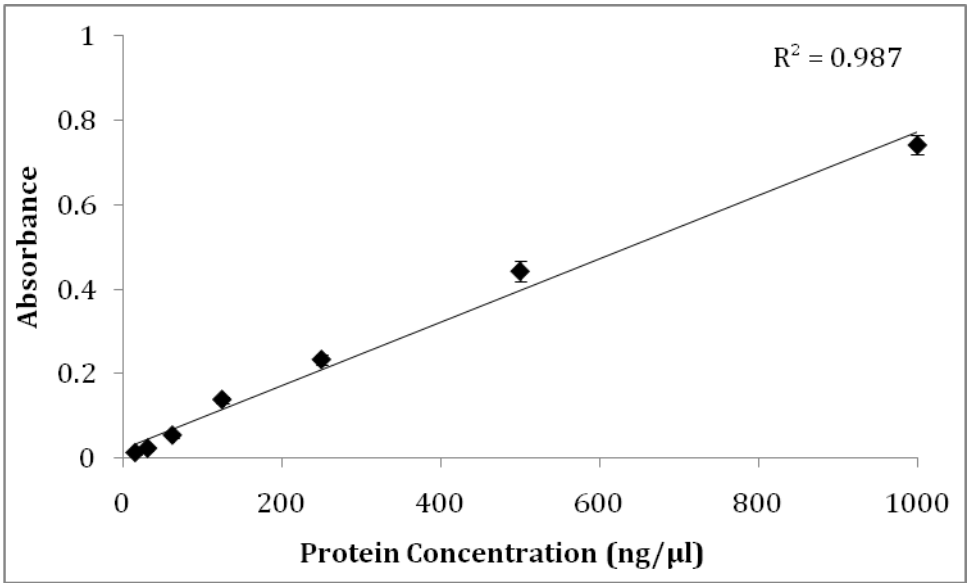


Figure 2.4: Example of a calibration curve produced using the BCA assay using BSA protein standards, analysed using a FLUOstar Optima Plate Reader.

2.4 DNA Amplification

2.4.1 PCR Reagents

A standard PCR reagent mixture was generated comprising the following reagents:

Reagent	Working Concentration
buffer [various]	1 x
bovine serum albumin (BSA)	0.2 µg/µl
deoxyribonucleotide triphosphates (dNTPs)	200 µM each of ATP, CTP, GTP & TTP
DNA polymerase [various]	0.1 units/µl
low melting temperature agarose*	Variable
magnesium chloride ($MgCl_2$)	1 mM
poly(vinylpyrrolidine) (PVP)*	0.01 % (w/v)
primers – forward & reverse	Variable
tween-20*	0.1 % (v/v)

Table 2.1: PCR reagents made up in purified water where * indicates optional reagents not included in all PCR experiments.

A number of different DNA polymerases were investigated for their suitability within the microfluidic system, including three Hot-Start *Taq* DNA polymerases (Table 2.2).

DNA polymerase	Manufacturer	Hot-Start Conditions
BIOTAQ™	Bioline, UK	-
AmpliTaq™ Gold	Applied Biosystems, UK	11 minutes at 95°C
FastStart *Taq*	Roche Applied Science, UK	2 minutes at 95°C
GoTaq®	Promega, UK	2 minutes at 95°C

Table 2.2: Properties of the different *Taq* DNA polymerases investigated.

Forensically relevant primer sets for DNA amplification were obtained from two sources. Firstly, an AmpFlSTR® SGM Plus™ kit [Applied Biosystems, UK] was used. This kit contains the primer sets for ten individual STR loci (D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01 and FGA) plus the Amelogenin sex marker in single solution. The kit also includes AmpliTaq™ Gold DNA polymerase and a PCR

reaction mixture which is under proprietary constraints but is known to contain: buffer, $MgCl_2$, dNTPs, BSA and sodium azide (NaN_3).

Secondly, custom-made primer sequences were obtained for nine forensically relevant loci that are present in both UK (AmpFlSTR® SGM Plus™ kit [Applied Biosystems, UK]) and US (PowerPlex® 16 System [Promega, US]) DNA profiling kits. Primers sequences from the PowerPlex® 16 System are not kept under proprietary constraints and so these sequences were used.[196] Primers were obtained with a fluorescent modification or 'tag' at the 5' end to enable visualisation of the PCR products [Eurofins MWG Operon, Germany]. The fluorescent tags used were 6-carboxyfluorescein (FAM) (excitation 495 nm, emission 520 nm), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE) (excitation 520 nm, emission 548 nm), *N,N,N',*N'-tetramethyl-6-carboxyrhodamine (TAMRA) (excitation 555 nm, emission 576 nm) and 6-carboxy-X-rhodamine (ROX) for the size standard (excitation 590 nm, emission 620 nm) (Figure 2.5).

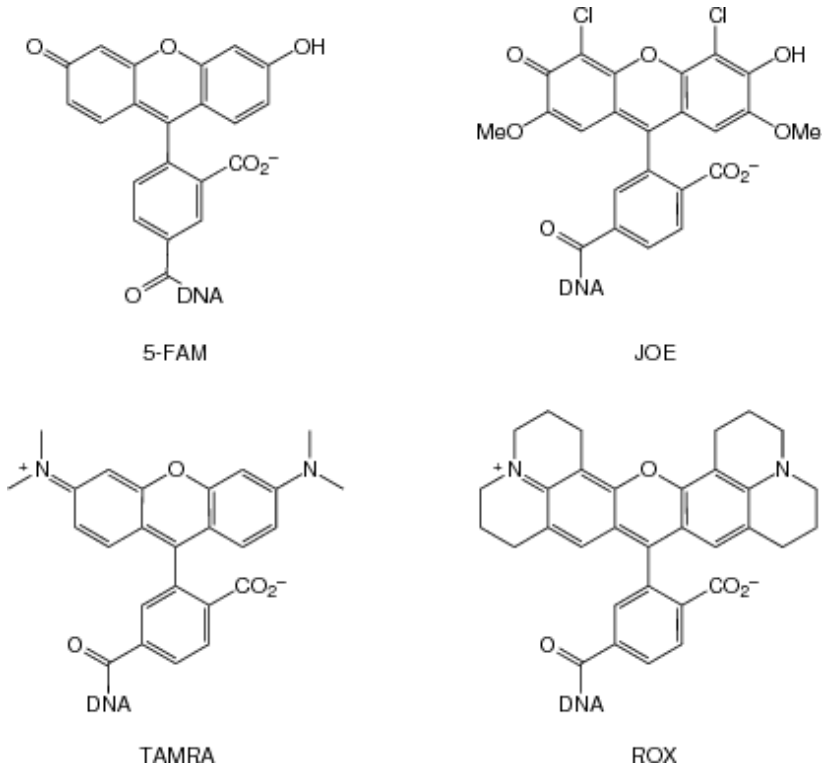


Figure 2.5: Structures of the different fluorescent tags used to label the primers and DNA size standard.

Locus	Chromosomal Location	Repeat Motif	5' Tag	Primer Sequence (5' – 3')
Amelogenin	X / Y	6 base pair deletion on X chromosome	JOE	F: CCCTGGGCTCTGTAAAGAA
				R: ATCAGAGCTTAAACTGGGAAGCTG
D3 S1358	3p21.31	[TCTG][TCTA]	FAM	F: ATGAAATCAACAGAGGCTTGC
				R: ACTGCAGTCCAATCTGGGT
D8 S1179	8q24.13	[TCTA][TCTG]	TAMRA	F: ACCAAATTGTGTTCATGAGTATAGTTTC
				R: ATTGCAACTTATATGTATTTTTGTATTTCATG
D16 S539	16q24.1	GATA	JOE	F: GTTTGTGTGTGCATCTGTAAGCATGTATC
				R: GGGGGTCTAAGAGCTTGTAAAAAG
D18 S51	18q21.33	AGAA	FAM	F: TTCTTGAGCCCAGAAGGTTA
				R: ATTCTACCAGCAACAACACAAATAAAC
D21 S11	21q21.1	Complex [TCTA][TCTG]	FAM	F: TGTATTAGTCAATGTTCTCCAGAGAC
				R: ATATGTGAGTCAATTCCCCAAG
FGA	4q28	CTTT	TAMRA	F: GGCTGCAGGGCATAACATTA
				R: ATTCTATGACTTTGCGCTTCAGGA
TH01	11p15.5	TCAT	JOE	F: GTGATTCCCATTGGCCTGTTC
				R: ATTCCTGTGGGCTGAAAAGCTC
vWA	12p13.31	[TCTG][TCTA]	TAMRA	F: GGACAGATGATAAATACATAGGATGGATGG
				R: GCCCTAGTGGATGATAAGAATAATCAGTATGTG

Table 2.3: Primer information, including sequences and fluorescent labels, for nine forensically relevant loci present in the PowerPlex® 16 System.[2, 196] (F = forward primer, R = reverse primer).

2.4.2 Conventional Thermal Cycling System

A conventional laboratory PCR instrument (TC-312 benchtop thermal cycler [Techne, UK]) was used to provide a technique to which DNA amplification on the microfluidic device could be compared. PCR samples were prepared, according to the PCR reagent mixture described above, in 0.2 ml DNase/RNase free, thin wall polypropylene PCR tubes [Alpha Laboratories, UK]. The PCR instrument used has a heated lid which prevents evaporation of samples during thermal cycling. An initial heating step at 95°C was used to both activate Hot-Start *Taq* DNA polymerases and to fully denature the DNA sample. Thermal cycling through temperatures for DNA denaturation (94°C), primer annealing (60°C) and DNA extension (72°C) was then carried out for up to 35 cycles (Figure 2.6). A final hold step at 60°C was used to ensure complete adenylation of all PCR products, preventing split peaks during analysis. The temperatures given here are for a standard DNA amplification reaction, if any deviation from these temperatures was required for individual experiments then details are provided in the subsequent chapters.

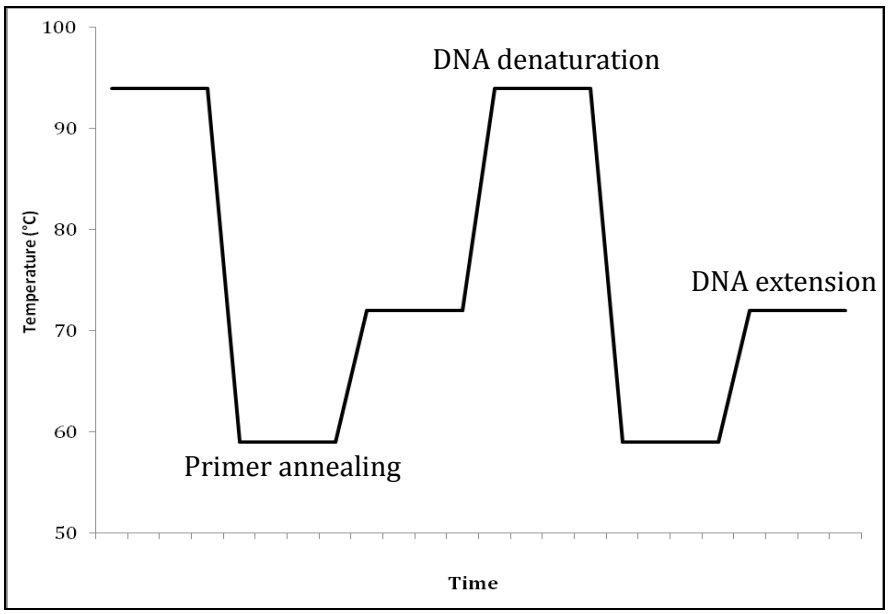


Figure 2.6: Schematic illustrating the DNA denaturation, primer annealing and DNA extension steps that form the thermal cycling profile for PCR DNA amplification.

2.4.3 Peltier Heating System

In order to carry out thermal cycling of the DNA amplification chamber on the microfluidic device, the use of Peltier heating systems was investigated. A Peltier is a thermoelectric solid-state device which is capable of heating and cooling based on the Peltier effect.[197] Heat is generated at the junction between two different conductors, in this case n-type and p-type semiconductors, when an electric current is applied, resulting in one side of the Peltier heating and the other side cooling (Figure 2.7). By changing the direction of the applied current the heating and cooling sides of the Peltier are reversed, enabling thermal cycling to be carried out.[198]

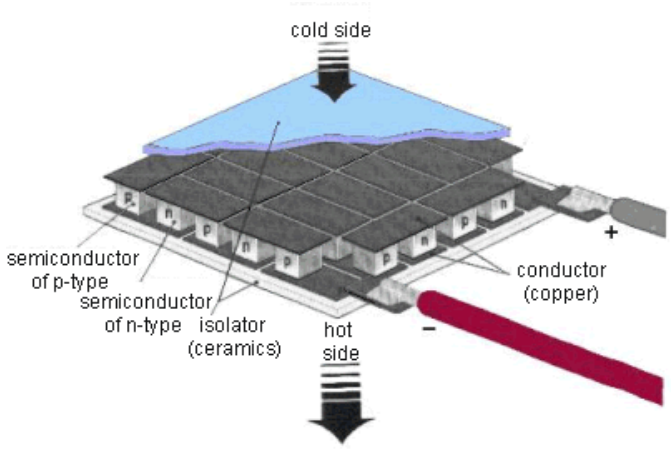


Figure 2.7: Schematic showing the composition of a Peltier device.[199]

The Peltier heating system was obtained from QuickOhm, Germany and developed in-house for the project requirements by Dr. Peter Docker. A convection heat sink was required to aid cooling of the Peltier, particularly for the primer annealing step. Temperature feedback control was provided using a K-type thermocouple. Figure 2.8 shows an image of the bench-top Peltier system used in initial studies, the same Peltier system set-up was also included in the portable integrated genetic analyser (see Chapter 2.7). The microfluidic device was placed on top of the Peltier and good thermal contact was ensured on the bench-top systems using thermal paste (Heat Sink Compound Plus

[RS Components Ltd., UK]) or on the integrated genetic analyser by spring loading the Peltier. The bench-top Peltier system was controlled used a custom-made Labview™ program, which also allowed the thermal cycling profile to be monitored (Figure 2.9).

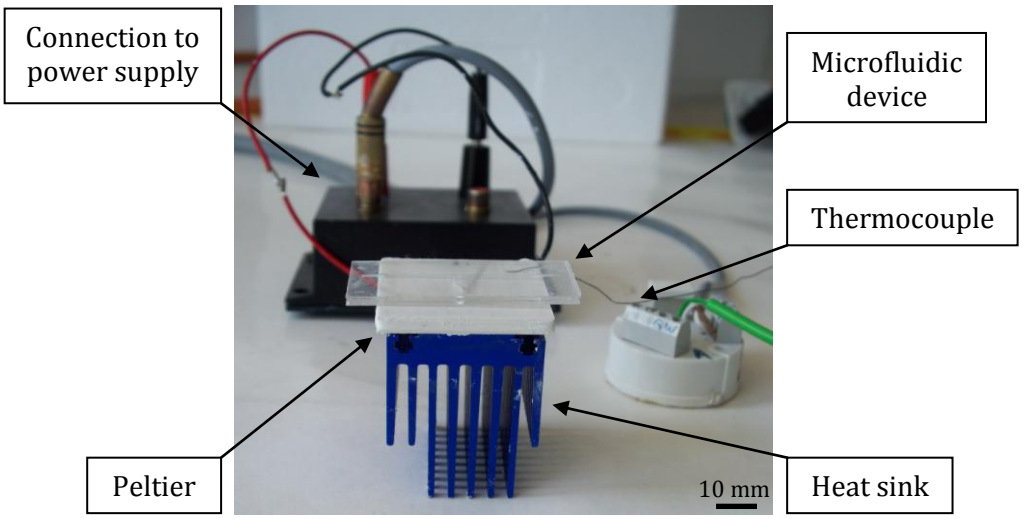


Figure 2.8: Photograph showing the components required for the Peltier heating/cooling system, including position of the microfluidic device.

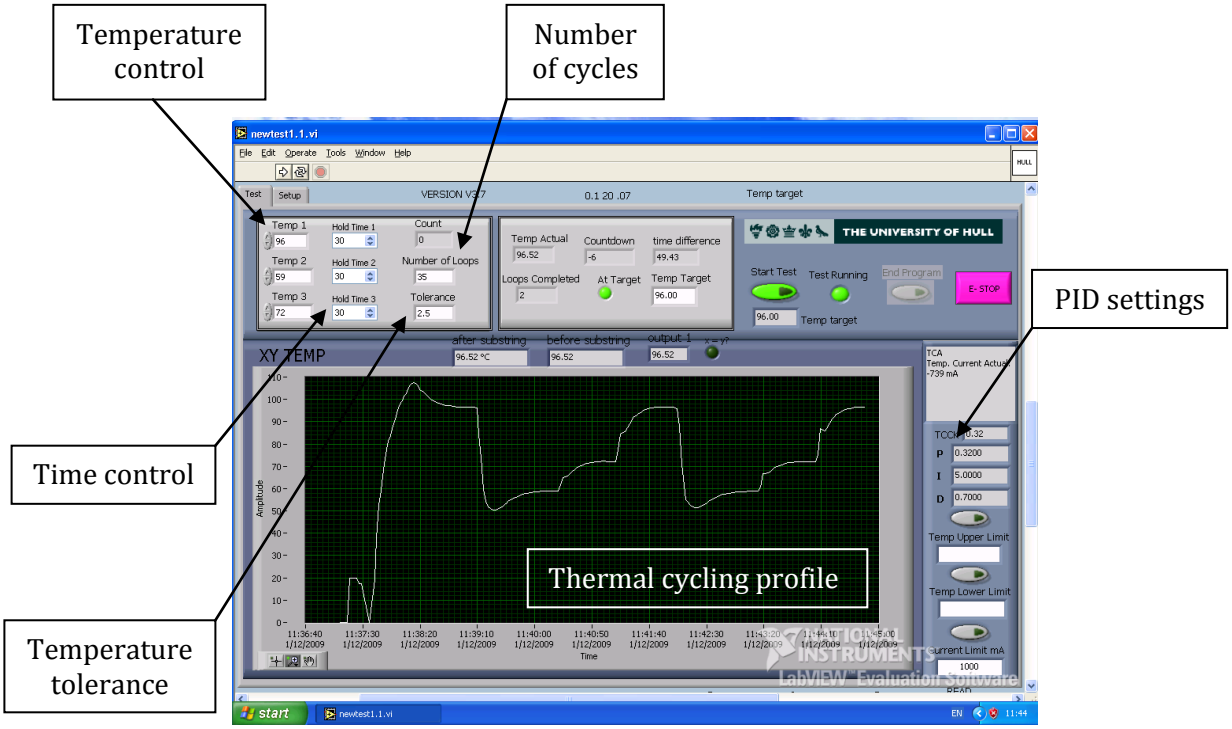


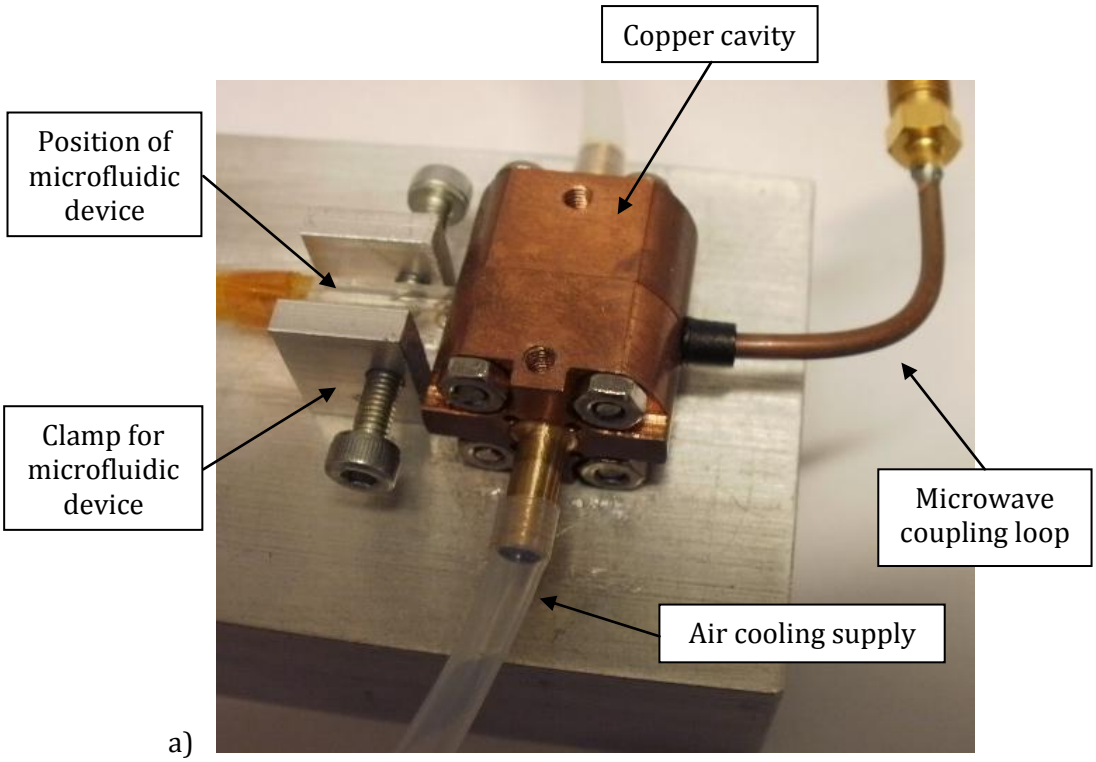
Figure 2.9: Screenshot showing Peltier control system with LabView™ interface. An example of the thermal cycling profile achieved using the system is shown.

2.4.4 Microwave Heating System

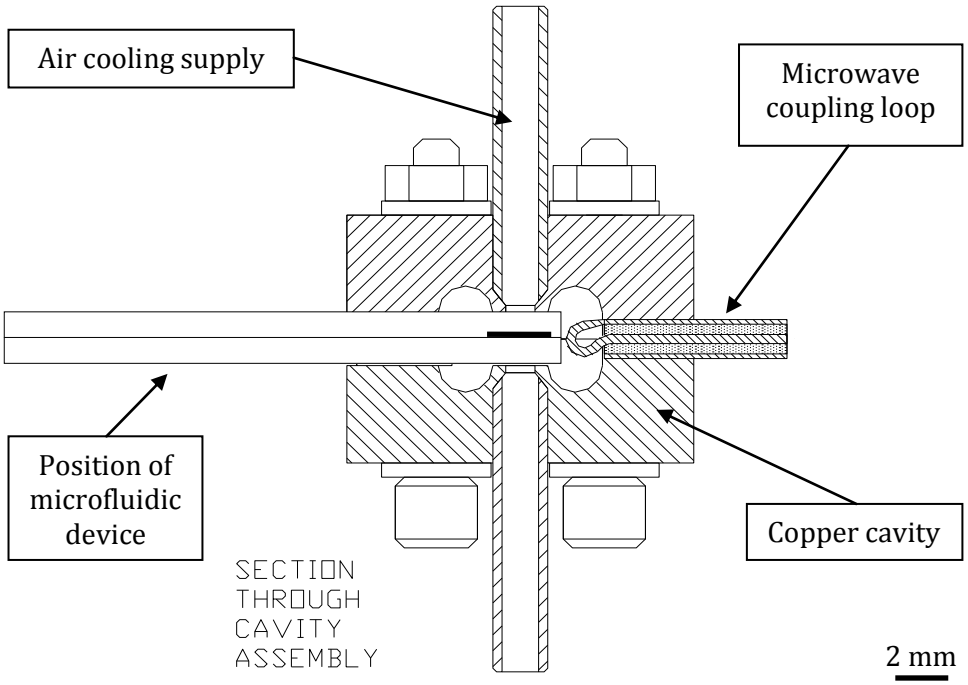
As an alternative to the Peltier heating system described above, a microwave-based thermal cycling system was commissioned from Dr. John Yelland [Richardsons, UK] which was also evaluated as a method for performing PCR DNA amplification. A re-entrant quasi-toroidal microwave cavity was developed in which the DNA amplification chamber on the microfluidic device was housed during the thermal cycling process.

Prior to performing thermal cycling using the microwave heating system it was necessary to determine the optimum frequency of the microwave cavity with the microfluidic device in place. The copper microwave cavity, which comes in two halves, can be separated to allow the microfluidic device to be placed opposite the microwave probe and then sealed in place by tightening the four screws present in each corner of the cavity (Figure 2.10).

The microwave probe was attached to an 8719D Network Analyzer [Hewlett Packard, UK] to allow the resonant frequency of the microwave cavity to be monitored. The Network Analyzer compares the frequency of the outgoing microwaves to those returning, resulting in a sharp peak on the monitor when the frequencies match, i.e. at the resonant frequency of the cavity (Figure 2.11). Tuning of the cavity to find the optimum frequency is achieved by tightening the four screws which hold the two halves of the microwave cavity together. In addition to tuning the cavity, this process also serves to hold the microfluidic device securely in place. As an added security measure a clamp external to the microwave cavity is used to secure the microfluidic device in place.



a)



b)

Figure 2.10: Cavity for performing microwave-based DNA amplification: a) Photograph and b) Schematic showing the set-up of the microwave cavity showing the positions of the microwave probe, air cooling supply and microfluidic device.

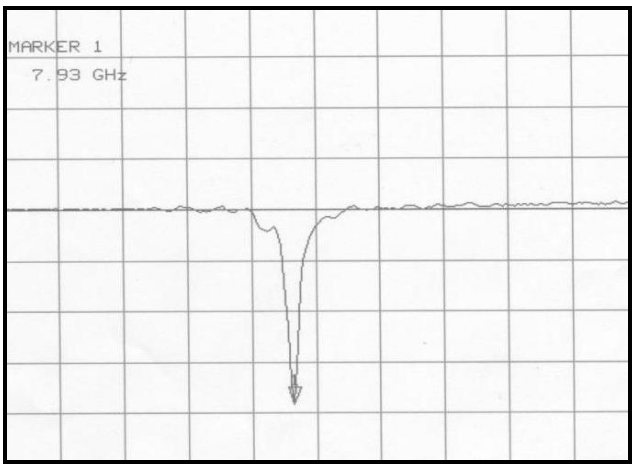


Figure 2.11: Graphical image from the Network Analyzer demonstrating the sharp peak obtained when the resonant frequency of the cavity has been achieved. In this example the optimum frequency is shown as 7.93 GHz.

Once tuned the microwave cavity is removed from the Network Analyzer and then attached to a VZM6991 series 8 – 18 GHz 20 W travelling wave tube amplifier (TWTA) [Communication & Power Industries, US] which acts as the microwave power source. By attaching the TWTA to a HP3850B signal generator [Hewlett Packard, UK] this enables microwaves to be generated at the optimum frequency, as previously determined. In order to achieve the different temperatures required for thermal cycling the power input was adjusted between 200 mW and 400 mW. Cooling was provided using compressed air, at a pressure of 4 bar, controlled by a solenoid and temperature feedback was achieved using a K-type thermocouple [Omega Engineering Ltd., UK]. The required temperatures and hold-times were programmed in using a purpose-built control system (Figure 2.12). In addition the control system allows real-time adjustment of the temperatures. Temperatures are monitored on a digital readout on the control system and also using a TDS210 Digital Storage Oscilloscope [Tektronix, Inc., US].

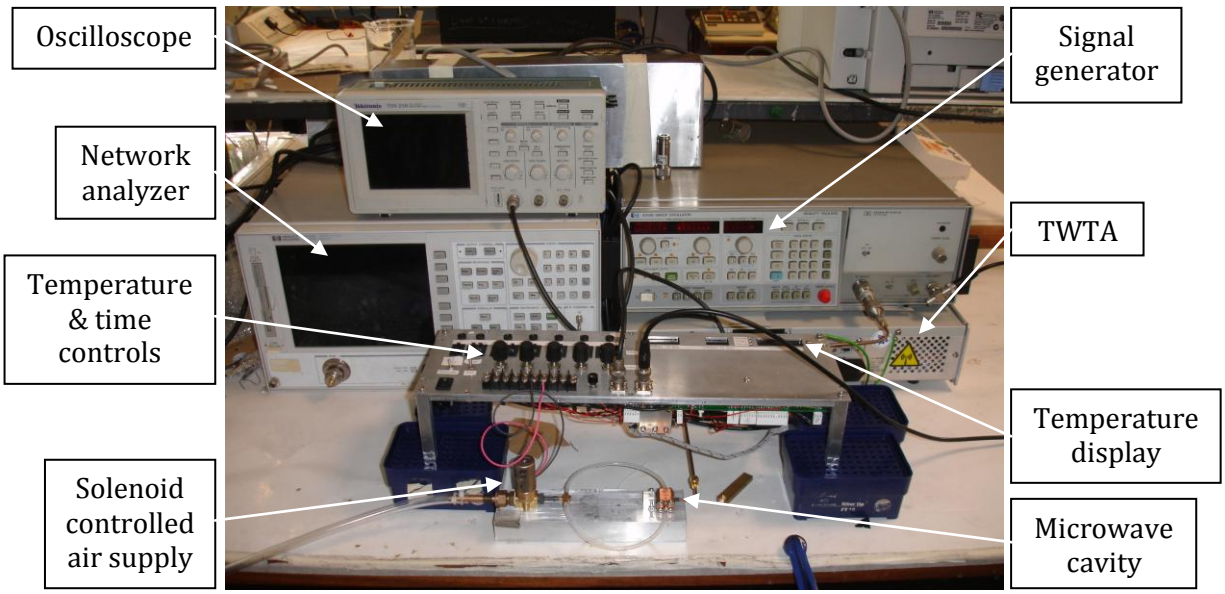


Figure 2.12: Photograph showing the control system for the microwave-based DNA amplification system, along with the Network Analyzer, TWTA and signal generator.

2.5 Analysis of PCR Products

2.5.1 Slab-Gel Electrophoresis

Slab-gel electrophoresis was performed using 2 % (w/v) agarose gels, prepared by dissolving 2 g agarose [Bioline, UK] in 100 ml 0.5x TBE buffer (0.09 M Tris-HCl [Sigma-Aldrich, UK], 0.09 M boric acid [Fisher Scientific] and 0.002 M EDTA in purified water) and allowing to solidify in a 15 x 15 cm gel tray. Once cooled, the gel was placed in a HU15 standard horizontal gel electrophoresis tank [Scie-Plas, UK] containing 0.5x TBE buffer prior to sample loading. The sample loading solution consisted of 2 µl of PCR product with 3 µl of 1x DNA loading buffer [Bioline, UK]. A 5 µl aliquot of DNA size standard, Hyperladder II [Bioline, UK] was also added to one of the wells in the gel. Samples were electrophoresed at 120 V until adequate separation was achieved. Gels were then stained using 0.5 µg/ml ethidium bromide [CLP, UK] in 0.5x TBE buffer for 20 minutes, then visualised using a UV transilluminator [Syngene, UK]. Gel images were recorded using Genescan software [Syngene, UK].

2.5.2 Capillary Gel Electrophoresis

Capillary gel electrophoresis was performed on an ABi310 Genetic Analyser [Applied Biosystems, UK]. Samples were prepared by mixing a 1 µl aliquot of PCR product with 0.5 µl of GeneScan™ 500 ROX™ DNA size ladder and 12 µl Hi-Di formamide [Applied Biosystems, UK]. The samples were incubated at 95 °C for 5 minutes in order to denature the DNA and then snap cooled on ice to maintain the DNA in a single-stranded form. Once the samples were loaded on to the ABi310 Genetic Analyser, electrokinetic injection was used to transfer the samples into the capillary electrophoresis column containing performance optimised polymer 4 (POP-4) [Applied Biosystems, UK] as the separation matrix. The PCR products undergo laser excitation and are detected fluorescently. The

inclusion of the DNA size ladder allows the size, in base pairs, of any peaks present in the electropherogram to be automatically assigned.

2.5.3 Real-Time Detection

In addition to the electrophoretic techniques described above for end-point analysis of PCR products, it is also possible to monitor the production of PCR products in real-time. To enable real-time monitoring of PCR product formation a modified version of the PCR protocol was carried out, with the addition of either an intercalating dye (PicoGreen™) or TaqMan™ style probes. A laser beam was directed at the DNA amplification chamber and pulsed on once every thermal cycle and the resulting fluorescence detected using a S2000 fibre optic spectrometer [Ocean Optics, UK] (Figure 2.13).

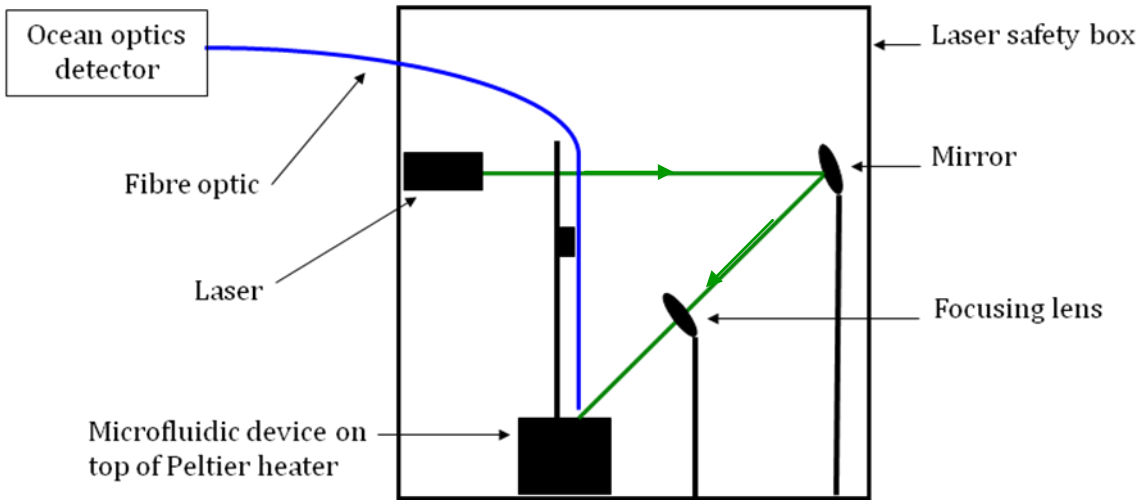


Figure 2.13: Schematic of the experimental set-up used to perform real-time PCR experiments.

For real-time experiments using an intercalating dye, 1 µl of concentrated PicoGreen™ solution was added to the PCR reagent mixture, allowing fluorescent detection of dsDNA.

PicoGreen™ was excited using a 488 nm laser and the resulting fluorescence monitored at 520 nm. For real-time experiments using TaqMan™ style probes were obtained for *Rattus norvegicus* GAPDH and CYP1A2 with FAM and JOE fluorescent tags which were excited using a continuous wave 488 nm solid-state laser [FCD488, JDS Uniphase Corparation, US] and the resulting fluorescence monitored at 518 nm and 548 nm respectively.

The latter experiments formed part of a supervised BSc student project where primary rat hepatocytes were induced using 3-methylcholanthrene, RNA was extracted using TRIzol® methodology and then reverse transcription (RT)-PCR was performed on the microfluidic device. Induction of primary rat hepatocytes [Abcellute, UK] was carried out by LGC, UK as part of the collaborative project. Briefly, 1 µM 3-methylcholanthrene added to the culture medium without serum and the cells induced for 24 hours. Both induced and non-induced primary rat hepatocytes (0.4×10^6) were stored in 1 ml TRIzol® reagent [Invitrogen, UK] at -80°C until required.

For the TRIzol® extraction homogenised samples were incubated at room temperature for 5 minutes prior to addition of 0.2 ml chloroform. Following 15 seconds of shaking the samples were incubated at room temperature for 2-3 minutes before being centrifuged at 12000 x g at 2-8°C. The aqueous phase was then transferred to new 1.5 ml microcentrifuge tube and 0.5 ml isopropanol added. Samples were then incubated at room temperature for 10 minute and centrifuged for a further 10 minutes at 12000 x g. The supernatant was then removed and the pellet washed with 75% ethanol, centrifuged at 7500 x g for 5 minutes at 2-8°C and then air dried for 10 minutes. Finally the pellet was dissolved in RNase free water by incubation at 60°C for 10 minutes.

A one-step RT-PCR reagent mixture was then produced comprising of: 1 µM forward primer, 1 µM reverse primer, 250 nM probe, 5 U M-MLV reverse transcriptase [Promega, UK], 1 x M-MLV reaction buffer, 1 mM $MgCl_2$, 400 µM each dNTPs, 0.2 µg/µl BSA, 0.01% (w/v) PVP, 0.1% (v/v) Tween-20, 1U Taq DNA polymerase and 0.5 µg RNA. Custom-made PCR primers and probes for GAPDH and CYP1A2 were obtained from

Eurofins MWG Operon, Germany (Table 2.4). Prior to performing standard PCR protocols, the reverse transcription step was carried out which required heating to 70°C for 5 minutes and then 37°C for 15 minutes.

Oligonucleotide	Sequence (5' – 3')
GAPDH Forward Primer	CAAGGTCATCCATGACAACTTTG
GAPDH Reverse Primer	GGGCCATCCACAGTCTTCTG
GAPDH Probe	FAM-ACCACAGTCCATGCCATCACTGCCA-BHQ1
CYP1A2 Forward Primer	TCCACATTCCCAAGGAGTGCT
CYP1A2 Reverse Primer	TAAGAAACCGCTCTGGGCG
CYP1A2 Probe	JOE-AGTGGCAGGTCAACCATGATGAA GCA-BHQ1

Table 2.4: Primer and probe sequences for performing real-time RT-PCR of GAPDH and CYP1A2.[200]

2.6 Portable Integrated Genetic Analyser

In order to perform DNA extraction, DNA amplification, capillary electrophoresis and fluorescence detection together on the microfluidic device, a control system was developed capable of integrating all the external components, such as the Peltier and power supplies, together. In collaboration with JLS Designs Ltd, Dr. Peter Docker produced a working prototype of a system which was capable of performing all the necessary operations for fully integrated genetic analysis (Figure 2.14). The system does not have the capabilities for hydrodynamic pumping of solutions but was designed to work using electro kinetic methods of sample and reagent movement. Thermal cycling is performed using a built-in Peltier heating system with an attached thermocouple for feedback. Electrokinetic movement is provided using four H10P (0 - 1 kV) and two H101P (0 - 10 kV) power supplies [EMCO, US]. The PCR products are detected fluorescently using a continuous wave 488 nm solid-state laser [FCD488, JDS Uniphase Corporation, US] and a diode pumped 561 nm laser [CL561, CrystaLaser®, US] for excitation, and two HR4000 spectrometers [OceanOptics, Inc., US] for detection. A touch-screen control panel interface enables access to set-up related experimental parameters, in both research and operator modes, run using custom-built software [JLS Designs Ltd., UK]. The research interface allows adjustable control of each of the individual process, while the operator interface simply has a 'GO' button reducing the complexity of operation.

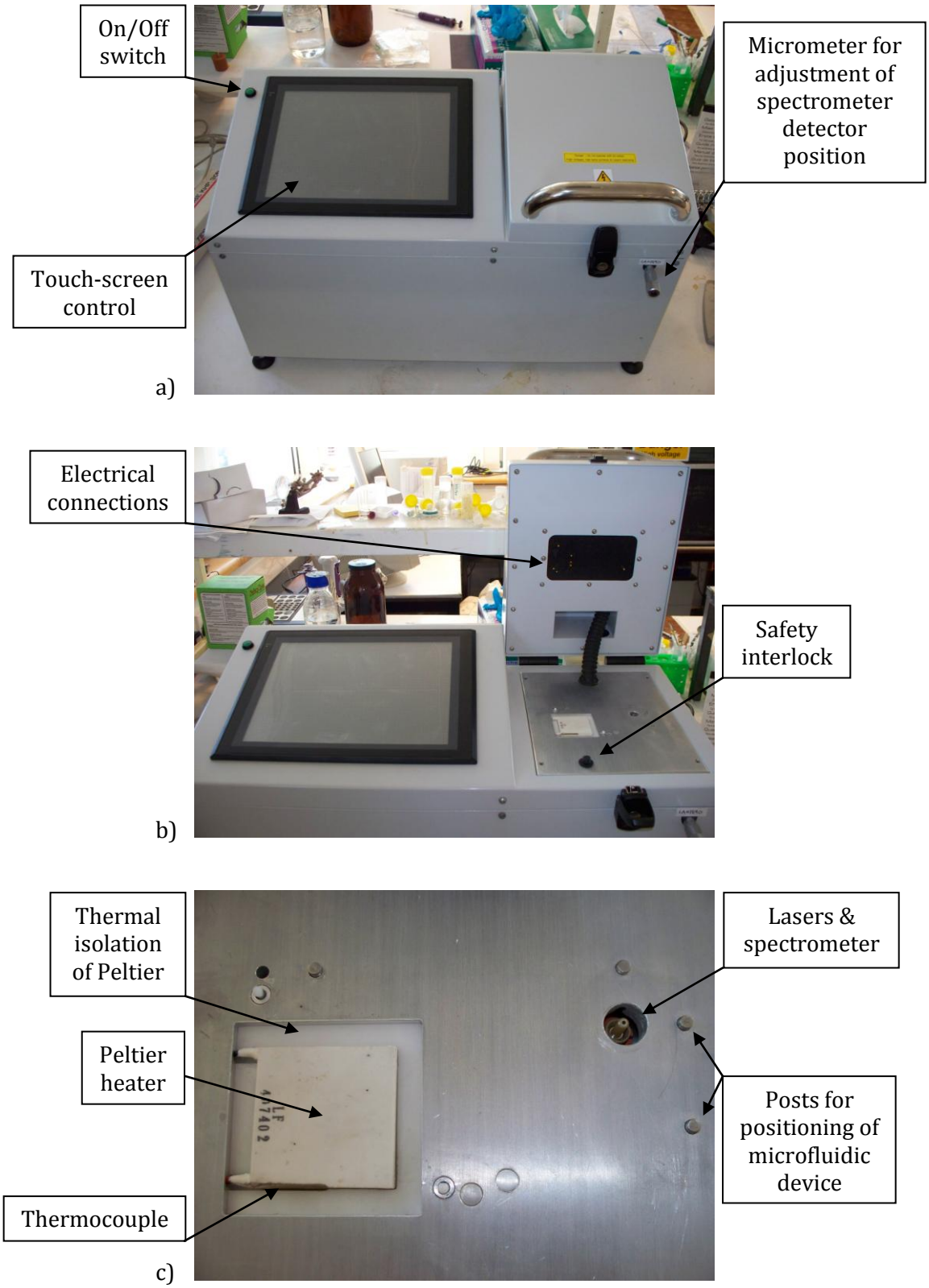


Figure 2.14: Photographs of the prototype portable integrated genetic analyser, highlighting important components where a) entire integrated genetic analyser, b) focus on the control panel and microfluidic device platform and c) close-up view of the components integrated into the microfluidic device platform.

3 Solid-Phase DNA Extraction using Hydrodynamic Pumping

3.1 Introduction

This chapter investigates solid-phase DNA extraction, using hydrodynamic pumping, on a microfluidic device. The two subsequent chapters will evaluate the performance of PCR DNA amplification in a microfluidic device and also the integration of the DNA extraction reported here with subsequent PCR analysis.

Following biological sample collection and cell lysis, DNA extraction is essential to reproducibly generate DNA of sufficient quantity and quality for PCR DNA amplification. As discussed in Chapter 1.2 there are a wide variety of techniques available for DNA extraction on both the macro- and micro-scale. As the ultimate aim of the project was to develop a microfluidic system for forensic genetic analysis, it is a consideration that biological samples for such applications are often of limited availability; it was therefore decided to use a solid-phase extraction (SPE) technique as this has the advantage of allowing pre-concentration of the DNA. DNA is immobilised onto the solid support while a wash step removes any contaminants and the DNA can then be eluted from the support in a small volume of liquid thus facilitating pre-concentration.

Of the two main SPE methodologies, ion exchange and silica-based, the latter enables the elution of DNA in a format which is more compatible with PCR. Using a silica-based solid-phase, DNA can be eluted in water or a low ionic strength buffer, whereas in ion exchange elution of DNA requires an increase in the alkalinity of the buffer solution as high as pH 10.6.[79] In addition, the chaotropic salts used to facilitate DNA adsorption in silica-based SPE also cause cell lysis eliminating the need to incorporate complex cell lysis techniques such as electroporation or ultrasonication.[37-41]

Hydrodynamic pumping was selected for use in the systems reported here as it is a well established technique for movement of solutions around microfluidic systems. Fluidic

connections can be provided using tubing and connectors to allow accurate control of flow rates of solutions using syringe pumps.

This chapter will report the evaluation of the use of different matrices for performing solid-phase DNA extraction using silica-based technology in order to achieve optimal performance on a microfluidic device. DNA extraction was optimised for silica beads, thermally-activated and photo-initiated silica-based monoliths as the solid support matrix so that maximal yields of DNA could be recovered during the elution process. A comparison of the different solid-phase methodologies was carried out to ensure that the most appropriate technique was incorporated into the final integrated genetic analysis system.

While DNA extraction in microfluidic devices has been previously reported in open channels, the efficiency is significantly less (39%) than when a porous solid-phase matrix is utilised (83%).[78] This not only increases the surface area available for DNA adsorption but also decreases the diffusional distance DNA molecules have to travel in order to become adsorbed onto a surface. The diffusion distance of a molecule is given in Equation 1.2.

Commercially available kits such as the QIAamp® DNA Micro Kit [Qiagen, UK] include carrier molecules to improve DNA yields during the elution phase and the applicability of including carrier RNA in the microfluidic DNA extraction system is evaluated. DNA extraction efficiencies were calculated using DNA of known concentration but the system was also tested using a range of biological sample types. Any microfluidic DNA extraction system developed for forensic purposes must be capable of accepting a variety of biological sample types that may be found at the scene of a crime as well as criminal justice reference samples. Analysis of the effectiveness of the DNA extraction methodology established was compared using both DNA quantification and PCR DNA amplification.

3.2 Experimental

3.2.1 Microfluidic Device Design for DNA Extraction

Microfluidic devices were manufactured by Dr. Steve Clark at the University of Hull, according to the protocol given in Chapter 2.1. DNA extraction chambers were etched to a depth of 100 µm, using the design shown in Figure 3.1. The volume of the DNA extraction chamber was 2.4 µl.

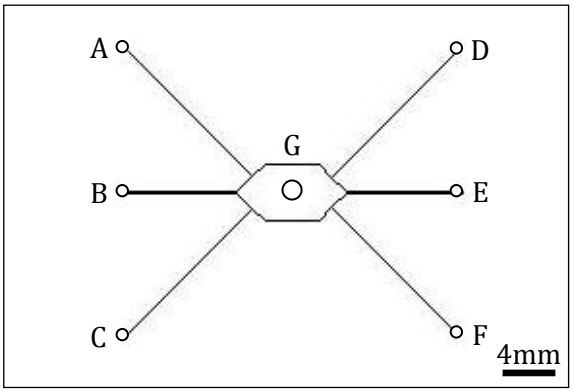


Figure 3.1: Schematic showing the microfluidic device for DNA extraction, including 360 µm ports (A-F) for addition of PEEK tubing. A 1 mm port (G) was included in the centre of the DNA extraction chamber to facilitate addition of the solid-phase matrix. All features are etched into the top plate to a depth of 100 µm.

3.2.2 Production of Solid-Phase Extraction Matrices

Three types of silica-based solid-phase extraction matrices were investigated: silica beads, photo-initiated monoliths and thermally activated monoliths.

Silica beads (irregular shaped, diameter 40-63 µm, surface area 550 m^2/g [227196, Sigma-Aldrich, UK]) were incubated overnight in 10 mM TE buffer then re-suspended in water.[48] The silica beads were then packed through the injection hole in the top plate

(Port G on Figure 3.1) and into the extraction chamber, producing a monolayer, and then the hole was sealed using Double Bubble Mix & Fix epoxy resin [Bondmaster®, UK]. The hexagonal geometry of the microfluidic device was designed such that the silica beads are held in place due to a keystone effect, confining the silica beads to the DNA extraction chamber (Figure 3.2).

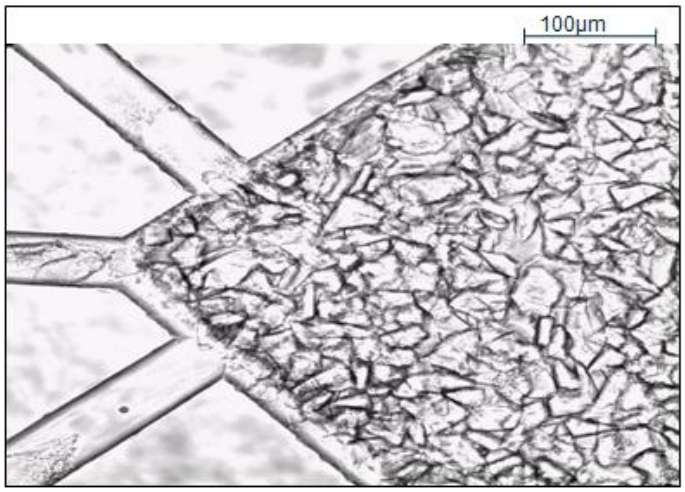


Figure 3.2: Microscope image of silica beads packed in the DNA extraction chamber.

Photo-initiated monoliths were produced from a mixture of monomer, porogen and photoinitiator. The monomer solution, 79 µl methacryloxypropyltrimethoxysilane (MPTMS) [Sigma-Aldrich, UK]) was activated by incubation with 21 µl 0.1 M hydrochloric acid [Fisher Scientific, UK] for 30 minutes with constant agitation. This produces the colloidal sol-gel precursor, through a series of hydrolysis and condensation reactions, required to produce the photo-initiated monolith (Equations 3.1 - 3.3).[201]

Hydrolysis: $Si(OR)_4 + 4\ H_2O \rightarrow Si(OH) + 4\ ROH$ **(Equation 3.1)**

Condensation: $2\ (RO)_3SiOH \rightarrow (RO)_3Si\text{-}O\text{-}Si(OR)_3 + H_2O$ **(Equation 3.2)**

$(RO)_4Si + (RO)_3SiOH \rightarrow (RO)_3Si\text{-}O\text{-}Si(OR)_3 + ROH$ **(Equation 3.3)**

The photoinitiator, Irgacure 1800 [Ciba Speciality Chemicals, UK], was added to the porogen, toluene [Sigma-Aldrich, UK], at 5 % total weight of monomer/porogen solution. As part of the study into DNA extraction efficiency using photo-initiated monoliths, a variety of monomer to porogen ratios were used, ranging from 10:90 to 50:50. The monomer and porogenic solutions were combined for 30 minutes with constant agitation. The resulting solution was then injected into the DNA extraction chamber and polymerised overnight by exposure to 405 nm UV light (20 mW/cm^2) [MJB-3 Mask Aligner, Karl Suss, UK] (Figure 3.3). Any unreacted solution, not exposed to UV, was removed by washing with ethanol.[201]

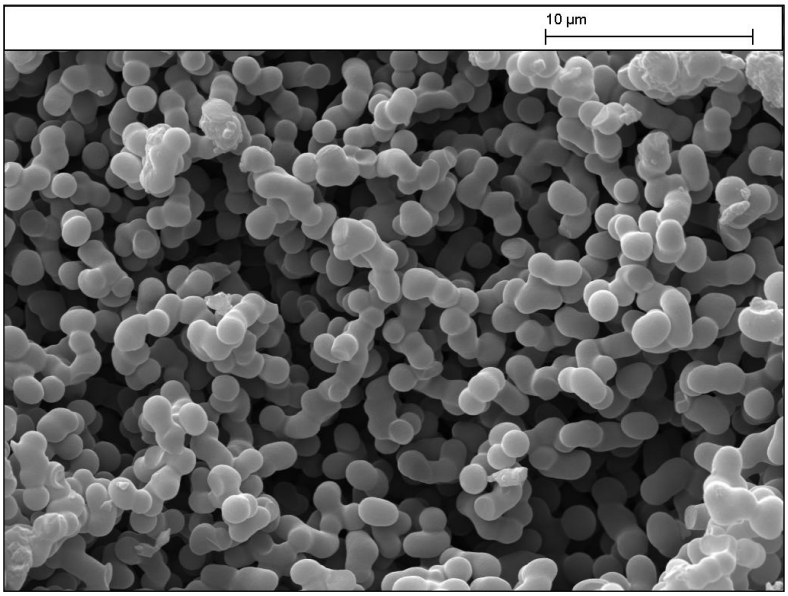


Figure 3.3: Scanning electron microscope image of the structure of a photo-initiated monolith at 10,000x resolution. In this example a monomer to porogen ratio of 30:70 was used.

Thermally-activated silica-based monoliths were produced by mixing potassium silicate solution (K_2SiO_3, 21 % SiO_2 and 9 % K_2O [VWR International, UK]) and formamide [Alfa Aesar, UK] in a 10:1 ratio [202]. In order to make sure 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 injected into the DNA extraction chamber until the glycerol solution had been visibly displaced from the chamber and then the microfluidic device was 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 (Figure 3.4). Any unreacted solution was removed by washing with ethanol prior to use for DNA extraction.

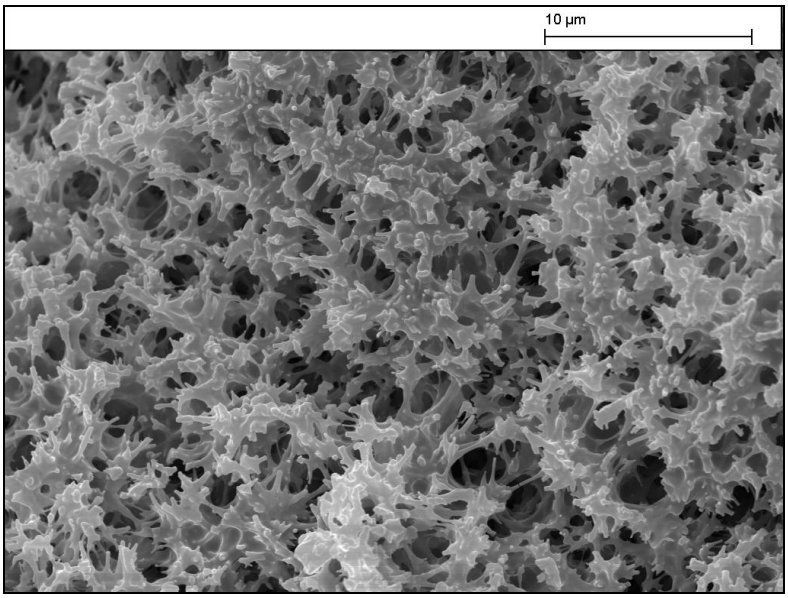


Figure 3.4: Scanning electron microscope image of the structure of the thermally-activated monolith at 10,000x resolution.

3.2.3 DNA Extraction using Hydrodynamic Pumping

DNA extraction on the microfluidic device was carried out using hydrodynamic pumping of reagents. Poly(etheretherketone) (PEEK) tubing (360 µm) was fitted into the ports that had been drilled into the microfluidic device and were held in place using epoxy resin. MicroTight® Adapters [Upchurch Scientific, UK] were used to connect 50 µl glass syringes to the PEEK tubing (Figure 3.5). The syringes were then connected to Baby Bee™ syringe drivers [Bioanalytical Systems Inc, UK] to allow control and adjustment of flow rates.

For individual optimisation experiments the type of solid-phase matrix, biological sample, reagent volumes and concentrations were varied as described throughout the results section, but in general the process for DNA extraction was as follows:

1) The solid-phase matrix was pre-conditioned with 10 mM TE buffer at 5 µl/min.

2) The biological sample in GuHCl loading solution was flowed over the solid-phase matrix, at 2.5 µl/min, resulting in DNA binding.

3) An 80 % (v/v) isopropanol solution was used to wash the solid-phase matrix at a flow rate of 5 µl/min.

4) The DNA was eluted in 10 mM TE buffer at 1 µl/min.

The steps described above were performed sequentially and, from the time of DNA addition to the microfluidic device, all solutions which had flowed over the solid-phase matrix were collected in 2 µl aliquots. These samples were then either analysed using PicoGreen® for DNA quantification or were used in PCR for DNA amplification.

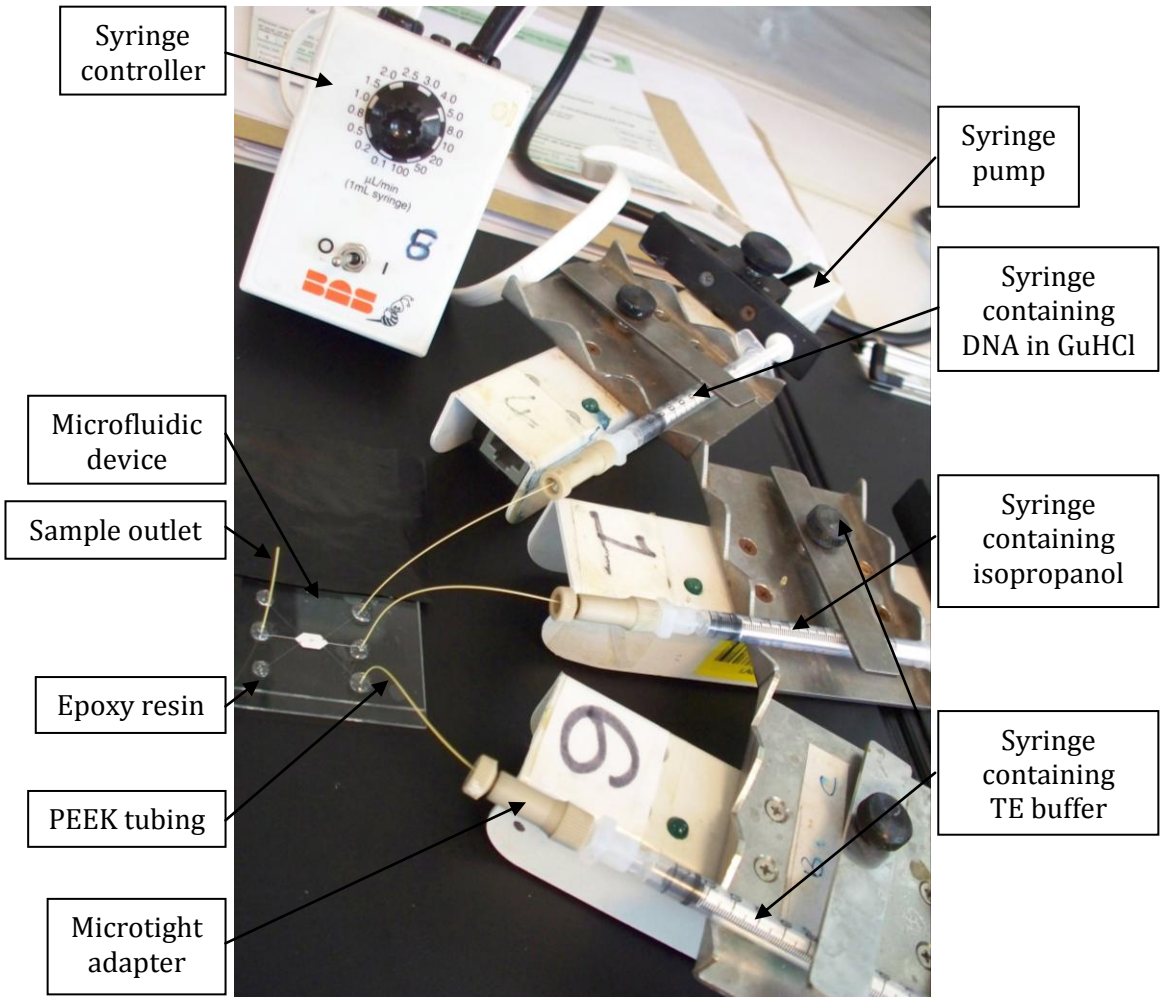


Figure 3.5: Photograph showing the set up of the microfluidic device and control apparatus for hydrodynamic pumping to perform DNA extraction.

3.3 Results & Discussion

3.3.1 Solid-Phase Pre-Treatment

Initial studies performing solid-phase DNA extraction on silica beads involved directly adding the DNA in GuHCl loading solution to silica beads that had been washed in purified water. The silica beads were then washed using 80% (v/v) isopropanol and the DNA eluted in 10 mM TE buffer. DNA quantification of the eluted fractions showed very low DNA concentrations (< 0.1 ng/µl when 25 ng was initially added). The silica beads were analysed by fluorescence microscopy, in the presence of PicoGreen™, at each stage of the DNA extraction procedure and it was demonstrated that whilst the DNA was successfully being adsorbed onto the solid-phase it was not being released during the elution step (Figure 3.6).

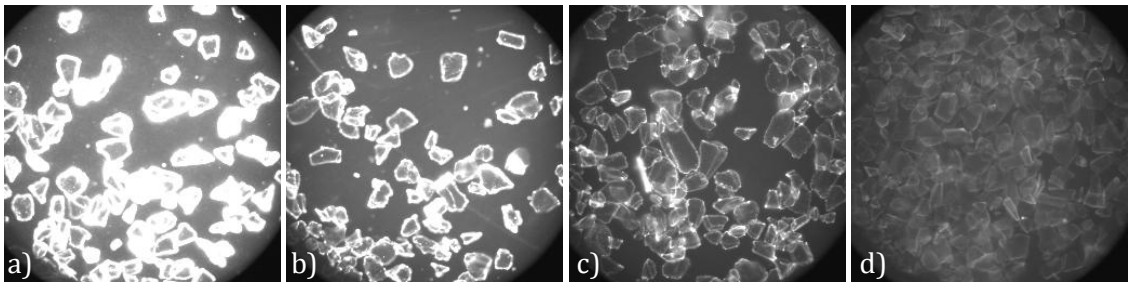


Figure 3.6: Fluorescent microscope images showing silica beads following DNA extraction. DNA remained adsorbed on the surface of the silica beads after the elution step, as visualised using PicoGreen® by fluorescent microscopy. Varying amounts of DNA were initially added during the DNA loading step where a) 10ng of DNA; b) 5ng of DNA; c) 1.25ng and d) negative control containing no DNA.

In order to eliminate this problem a pre-treatment of the solid-phase, prior to addition of DNA, was investigated by incubation with 10mM TE buffer (pH6.7) at a flow rate of 5 µl/min.[48] It was found that the optimal incubation time, i.e. that which resulted in the highest DNA yield during the elution phase, varied depending on the type of solid-phase

used (Figure 3.7). For the thermally-activated monoliths optimum DNA yields were produced when a 30 minute incubation step was used, for the photo-initiated monoliths 60 minutes incubation was required and for the silica beads an overnight incubation was necessary. Following TE treatment, the solid-phase could be used immediately for DNA extraction or washed with distilled water and retained for later use. For all subsequent experiments the optimum incubation time, depending on the silica matrix, was used prior to DNA extraction experiments being performed.

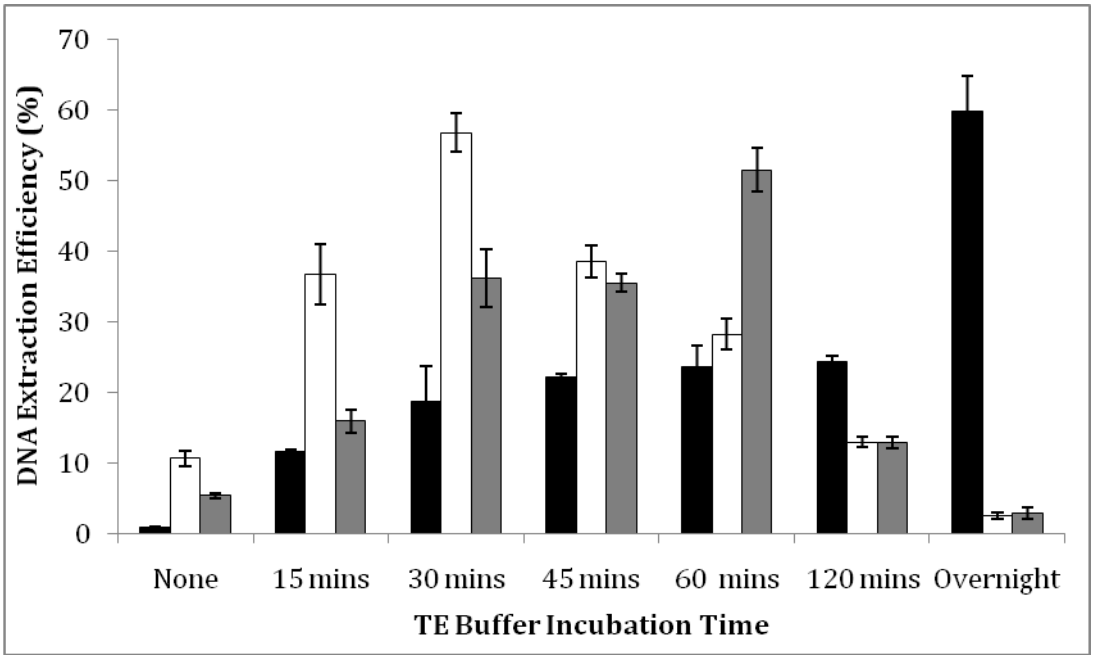


Figure 3.7: Pre-treatment incubation times with 10 mM TE buffer of the three different solid-phase matrices; silica beads (■), thermally-activated monoliths (□) and photo-initiated monoliths (▣), compared to DNA extraction efficiency. Error bars represent the standard deviation (n = 3).

Incubation of the silica surfaces with a buffer solution allows time for equilibration, with a fully hydroxylated silica surface having a theoretical pK_a of 7.1.[203] By using a buffer with a pH below that of the pK_a of the surface silanol groups, there is increased protonation and therefore a decrease in surface charge. This reduces the electrostatic repulsion between the DNA and the silica and so aids the formation of hydrogen bonds which allow the DNA

to bind to the surface.[48] During the elution phase, in a low ionic strength buffer, these hydrogen bonds are broken and the DNA goes back into solution. While the theoretical pK_a of silica is 7.1, differences in the manufacturing process can alter this value, which it is hypothesised may account for the differences in incubation time required of the three different types of silica matrix.

3.3.2 Optimisation of Guanidine Hydrochloride Concentration

DNA adsorption to a silica surface is facilitated by the use of high ionic strength chaotropic salts, which fulfil a variety of functions as described in Chapter 1.3.3. The effect of the concentration of the chaotropic salt, guanidine hydrochloride, on DNA extraction efficiency investigated. Silica beads, which had been pre-treated overnight in 10 mM TE buffer, were used as the solid-phase. A range of GuHCl concentrations, up to 6 M, were evaluated for use as the DNA binding solution using the methodology described in Chapter 3.2.3. Maximum DNA yields during the elution phase were found to be achieved using a 5 M GuHCl solution and so this was used in all subsequent experiments (Figure 3.8).

This result fits in well with published literature where GuHCl concentrations used in silica-based microfluidic devices range from 3 M to 8 M.[31, 54] Initially, increasing the GuHCl concentration resulted in an increase in DNA yield during the elution phase up to 5 M. One of the mechanisms by which the binding solution works is to reduce the negative potential at the silica surface which in turn decreases electrostatic repulsion between the DNA and silica thus aiding binding.[47] Increasing the concentration of the binding solution will cause a more significant decrease in electrostatic repulsion enabling more effective binding of DNA to the silica. In addition, increasing the concentration will form more hydrated ions and dehydrate the surface of the silica and DNA more which promotes DNA binding. Also there will be a threshold of GuHCl concentration which is required to achieve denaturation of the dsDNA and so enable hydrogen bonding of the exposed bases with the silica.[48] As

there is a significant increase in DNA yield using just a 1 M GuHCl solution compared to the negative control not containing GuHCl it would appear that this is sufficient to enable a certain degree of DNA denaturation and binding to the silica surface.

However, when a GuHCl concentration above 5 M is used in the binding solution the DNA yield during the elution step was found to decrease. It was hypothesised that when using very high ionic strength solutions, binding of DNA to the silica is in fact too strong and that while the binding process is very efficient, the elution of the DNA is more difficult and so reduced yields are obtained.

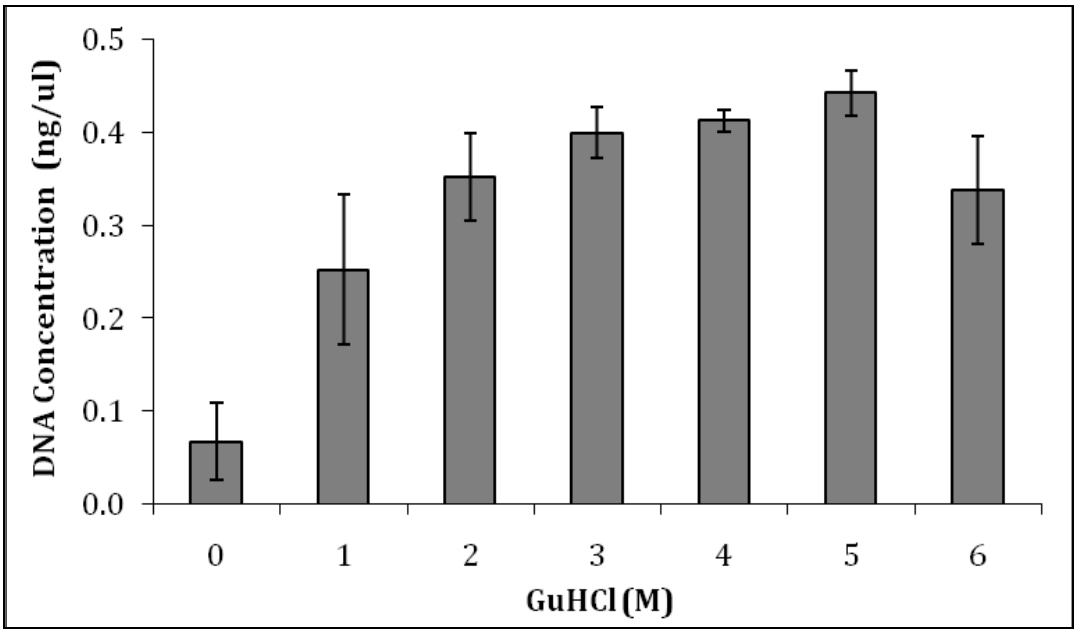


Figure 3.8: Effect of GuHCl concentration on DNA extraction efficiency. A range of GuHCl concentrations were tested for the loading of DNA onto the solid-phase matrix. The DNA concentrations yielded during the elution phase were determined using PicoGreen™ analysis. Each experiment was carried out in triplicate with standard deviations shown on the graph.

3.3.3 DNA Extraction using Silica Beads

Silica beads were the first to be evaluated as the solid-phase for DNA extraction, as they are often found as the packing material in commercially available DNA extraction kits, and they can be incorporated into microfluidic systems with relative ease (Chapter 1.3.4). Silica beads were held in place within the microfluidic device using tapered chamber geometry, as this could be easily incorporated into the manufacturing process, trapping the beads through the 'keystone' effect (Figure 3.2). In order to allow the DNA extraction efficiency of the system to be calculated (Equation 2.1), DNA of known concentration was used. This DNA was produced using a QIAamp® DNA Micro Kit [Qiagen, UK] to extract DNA from a saliva sample and then quantified using PicoGreen™ [Invitrogen, UK].

Initial studies showed the average DNA extraction efficiency using this system to be approximately 59%, however there was large variability in the results obtained with a standard deviation of 39%. It was hypothesised that the depth of the extraction chamber may allow solutions to flow over the top of the beads reducing contact between the mobile and stationary phases. In order to investigate whether this may be the case, the flow of solution was stopped within the DNA extraction chamber in order to allow greater interaction between the mobile and stationary phases. DNA extraction was carried out according to the method given in Chapter 3.2.3, using silica beads which had undergone overnight incubation in 10 mM TE buffer and a 5 M GuHCl solution for DNA binding. By stopping the flow for 10 minutes during the DNA binding step, loss of DNA during the wash step was minimised, resulting in higher yields during the DNA elution phase (Figure 3.9).

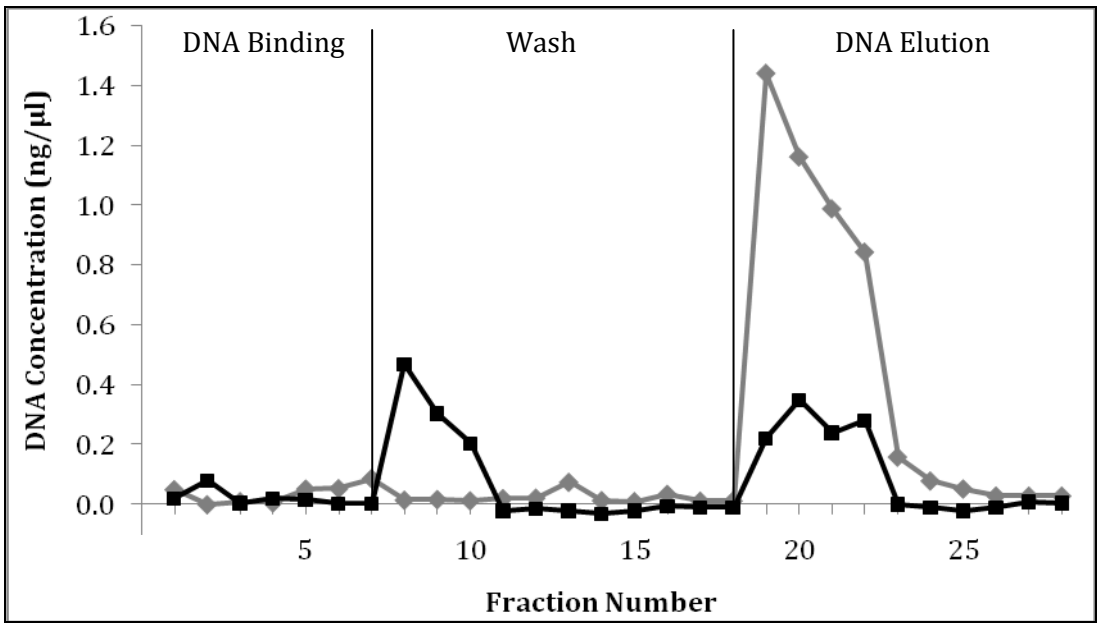


Figure 3.9: Effect of including a stop-flow step on DNA yield during the elution phase. DNA extraction profiles, with (—◆—) and without (—■—) the inclusion of a 10 minute stop-flow step during the DNA binding phase of the procedure.

As the DNA remains in the extraction chamber longer, more is adsorbed on to the silica beads, leaving less remaining in solution that can be washed away. Stopping the flow of solutions during the wash and elution steps made little difference to the recovery of DNA during the elution phase. It is therefore achieving maximum DNA binding that is the most crucial step in obtaining high DNA yields during the elution phase.

Preliminary experiments used a 10 minute hold of the DNA binding solution to produce more efficient DNA yields during the elution phase. However, when developing a portable system, speed of analysis is an important consideration and so a range of hold times were investigated to see if a reduction could be made whilst maintaining increased DNA adsorption. A hold-time of 2 minutes or greater was required to prevent loss of the DNA during the wash step (Figure 3.10).

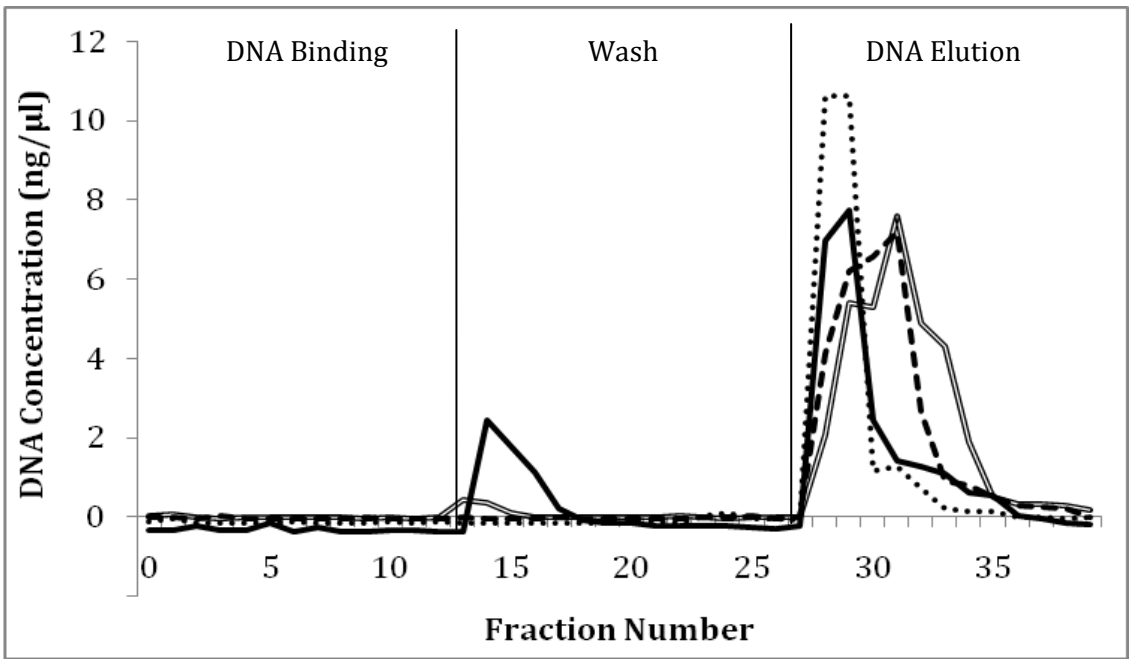


Figure 3.10: Effect of stop-flow hold-time on DNA yield during the elution phase. DNA in GuHCl solution was added to the DNA extraction chamber and the flow was stopped for either 5 minutes (·····), 2 minutes (- - - -), 1 minute (═══) or continuously flowed (——).

The longer the hold-time during the DNA binding phase of the reaction, the more DNA is collected during the elution phase, thus making the DNA extraction efficiency greater. A compromise between speed and efficiency of analysis indicates that 2 minutes is the optimum hold-time.

In order to try and reduce the overall time of the DNA extraction procedure further, the minimum volume of 80% (v/v) isopropanol wash required to remove all protein contamination was analysed. The protein content of sequentially collected wash fractions was analysed using a BCA assay. Using saliva samples it was shown that the majority of the proteins are removed in the first 10 µl fraction of the 80% (v/v) isopropanol wash (Figure 3.11). Using a flow-rate of 5 µl/min means that the wash step can also be completed in 2 minutes.

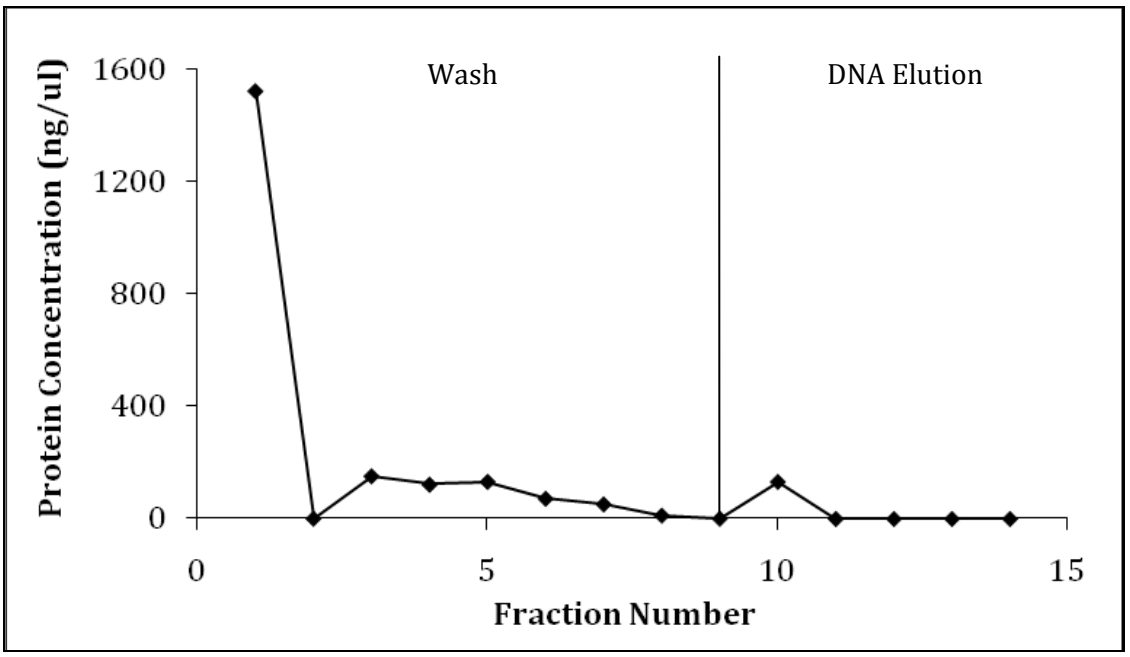


Figure 3.11: Analysis of the protein content of the wash phase. Saliva samples were added directly to the GuHCl binding solution and DNA extraction was performed according to the methodology given in Chapter 3.2.3. Sequentially eluted 10µl fractions were collected and subjected to BCA analysis.

The overall optimised protocol for performing DNA extraction using silica beads can be summarised as:

- Overnight incubation of the silica beads with 10 mM TE buffer at 5 µl/min.
- DNA loaded onto silica beads using a 5 M GuHCl solution at 2.5 µl/min, including a 2 minute stop-flow.
- Silica beads washed using a 10 µl volume of 80% (v/v) isopropanol at 5 µl/min.
- DNA eluted from the silica beads using 10 mM TE buffer at 1 µl/min.

The DNA extraction efficiency yielded, using the above optimised conditions, was found to be 77% ± 32%, compared to commercial kits which have been reported to have DNA

extraction efficiencies of approximately 49% ± 2% for pre-purified human genomic DNA.[75] However, such commercial kits have been optimised to accommodate much larger sample volumes and so cannot provide a direct comparison. Of the silica bead-based microfluidic DNA extraction systems reported in the literature, DNA extraction efficiencies range from 30 – 84% for pre-purified λ-phage DNA.[54, 65] The results presented here fit well within this range for purified DNA samples. The use of silica beads for DNA extraction from a range of biological samples will be discussed in Chapter 3.3.7.

However, even with the use of the stop-flow technique, DNA extraction efficiencies using silica beads as the solid-phase exhibited a dramatic lack of reproducibility, as can be seen by the standard deviation observed. This can be attributed to uneven packing of the system on addition of silica beads and a non-uniform flow of solutions through the packed system. In order to try and overcome these problems silica-based monoliths were investigated.

3.3.4 DNA Extraction using Photo-Initiated Monoliths

The use of sol-gels or monoliths as the solid-phase for DNA extraction within microfluidic devices has been reported to give greater reproducibility than bead-based systems (Chapter 1.3.4). In order to accurately control positioning of the solid-phase within the microfluidic device, the use of photo-initiated monoliths were investigated (see Chapter 3.2.2). By including a photo-initiator in with the monomer and porogen, polymerisation by UV exposure can be confined to specific regions within the microfluidic device, i.e. the DNA extraction chamber, using a photomask. The structure of the monolith can be controlled by varying the monomer (MPTMS) to porogen (toluene) ratio, which affects the pore size and therefore surface area available for DNA adsorption.

Preliminary studies to determine the optimal monomer to porogen ratio were, for high throughput and ease of microscopic analysis, carried out in 0.6 mm i.d. glass capillaries.

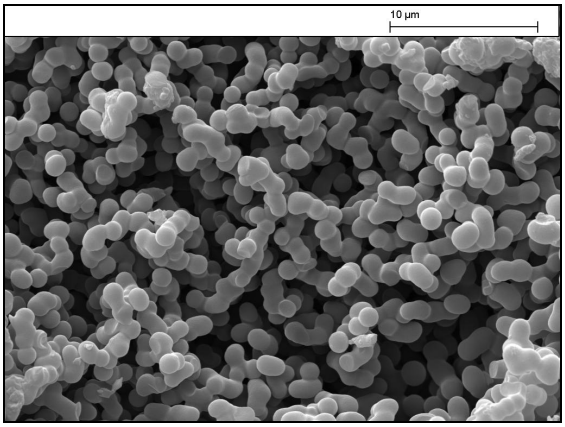
Photo-initiated monoliths were prepared, according to the protocol given in Chapter 3.2.2, using varying monomer to porogen ratios ranging from 10:90 to 50:50. DNA extraction was then performed using the methodology described in Chapter 3.2.3, including a 60 minute pre-treatment of the monoliths with 10 mM TE buffer. Increasing the amount of monomer increased the DNA yield during the elution phase, with an optimum ratio of 50:50 monomer to porogen. Increasing the amount of monomer creates a larger surface area for DNA adsorption, as evident from the SEM images of monoliths at different ratios (Figure 3.12).

However, when the photo-initiated monoliths were produced in the microfluidic device at the ratio of 50:50 monomer to porogen, the back-pressure produced in the system was too high to enable flow of solutions through the monolith. As can be seen from Figure 3.12c, the structure of the monolith is less porous and so this prevents flow of solutions through the system. Therefore the ratio had to be reduced to 30:70 monomer to porogen for use in the DNA extraction chambers on the microfluidic system to provide a compromise between maximum surface area for DNA adsorption and acceptable back-pressures to facilitate hydrodynamic pumping of solutions through the system.

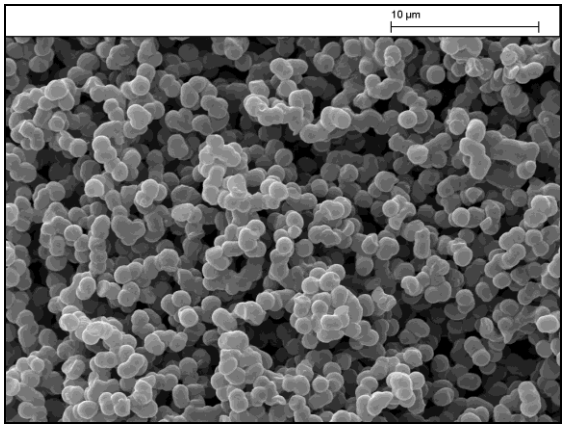
Using a monomer to porogen ratio of 30:70 and a pre-treatment with 10 mM TE buffer for 60 minutes, an evaluation of DNA extraction efficiency was carried out. Disappointingly the average DNA extraction efficiency of the photo-initiated monoliths was only 24% ± 5% (as summarised in Figure 3.14). There are a number of possible explanations for this, one of which is that incomplete polymerisation may be occurring due to poor penetration of the UV light through the glass microfluidic device. Alternatively, it is possible that by using a highly cross-linked organic polymer matrix, a proportion of the silica binding sites may be buried within the structure and therefore are inaccessible for the DNA. To overcome this issue the polymerised monolith can be modified, for example with TMOS, to increase the number of surface silica binding sites.[75] However, this further increases the complexity of producing a photo-initiated monolith within a microfluidic environment. As

the ultimate aim is to produce single use microfluidic devices for genetic analysis, the manufacturing process needs to be as simple as possible for mass production to be feasible option.

(a)



(b)



(c)

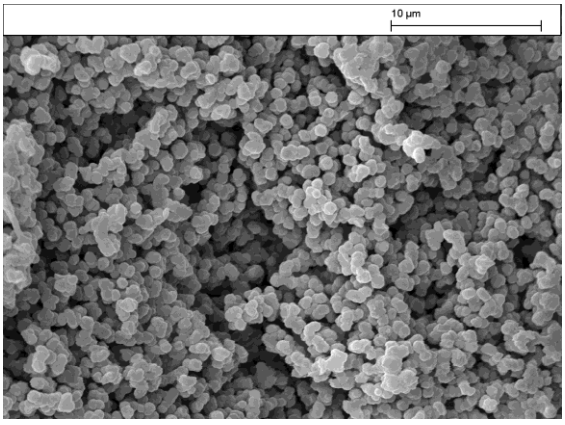


Figure 3.12: **Scanning electron micrographs of photo-initiated monoliths prepared at different monomer to porogen ratios; a) 30:70, b) 40:60 and c) 50:50 (Images courtesy of Mr. Tony Sinclair, University of Hull).**

3.3.5 DNA Extraction using Thermally Activated Monoliths

Previous work at the University of Hull has used thermally activated porous silica monoliths for the development of micro-reactor technology.[202] It was investigated whether these simple to produce silica monoliths could be used be used as the solid-phase for DNA extraction purposes. A mixture of potassium silicate and formamide, at a ratio of 10:1, was thermally cured at 90°C overnight to produce monoliths with optimum flow characteristics. Decreasing the temperature for curing resulted in smaller pore sizes and high back pressures in the system and therefore resulted in restricted flow of solutions through the monolith. Conversely if the temperature was too high then larger pores are produced ultimately leading to cracking of the monolith allowing solutions to flow round the periphery of the monolith and not through it, drastically reducing the surface area available for DNA extraction. Scanning electron microscopy (SEM) was used to examine in detail the structure of the monolith created using this method. As shown in Figure 3.4 the monolith has a large surface area with a good pore network preventing problems with high back pressure.

Initially it was found that thermally activated monoliths were not as easy to control with respect to their positioning within the microfluidic device as the photo-initiated monoliths. However a method was developed whereby, if the surrounding channels were filled with glycerol during the curing process (see Chapter 3.2.2) then the monolith solution could be confined to the DNA extraction chamber enabling precision placement (Figure 3.13).

Due to the more reproducible manner in which the thermally activated monolith can be formed in the microfluidic device, compared to the packing of silica beads, it was found that the inclusion of a stop-flow step during DNA binding was not necessary. Apart from this one alteration, the DNA extraction procedure was exactly the same for the silica monolith as the silica beads and resulted in an average extraction efficiency of 82% ± 6% (as summarised in Figure 3.14). Thermally activated monoliths have so far found limited

application in microfluidic systems, with the choice of monomer proving crucial for obtaining maximum DNA yield, for example, a TEOS monolith has an average DNA extraction efficiency of 33% efficiency [67] compared with a TMOS monolith which is capable of 85% efficiency when extracting phage DNA.[73] The use of potassium silicate as the monomer, as presented here, offers a simple to produce alternative to the use of TMOS thermally activated monoliths with comparable DNA extraction efficiency.

Along with the increase in DNA extraction efficiency, the use of silica monoliths increases the reproducibility of the extraction process, as compared to the use of silica beads. The ability of the system to produce reproducible results is important when dealing with forensic samples as any such system would be required to undergo stringent validation procedures prior to use.

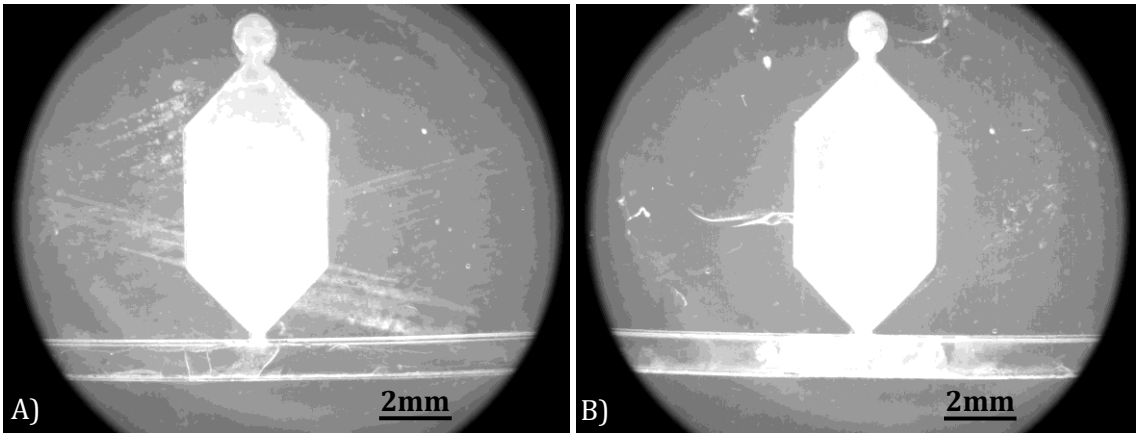


Figure 3.13: Photographs comparing the positioning of thermally activated monoliths with (A) and without (B) the use of glycerol.

3.3.6 Comparison of Solid-Phases for DNA Extraction

A comparison was made of the three types of solid-phase (silica beads, thermally activated potassium silicate monolith, photo-initiated MPTMS monolith) to determine the most suitable for inclusion within the microfluidic device. The use of silica-based monoliths leads to greater reproducibility within the system, as compared to silica beads. This is important for forensic applications as any potential portable DNA analyser would have strict validation requirements. In addition, the use of thermally activated monoliths greatly increases the efficiency of the DNA extraction which has considerable advantages in forensics. Samples found at the scene of a crime are often in low abundance so the more DNA that can be extracted from a sample the better, and also due to the nature of the samples there is not the option of going back to collect a second sample if required. A summary of the DNA extraction efficiencies of the different methodologies is shown in Figure 3.14.

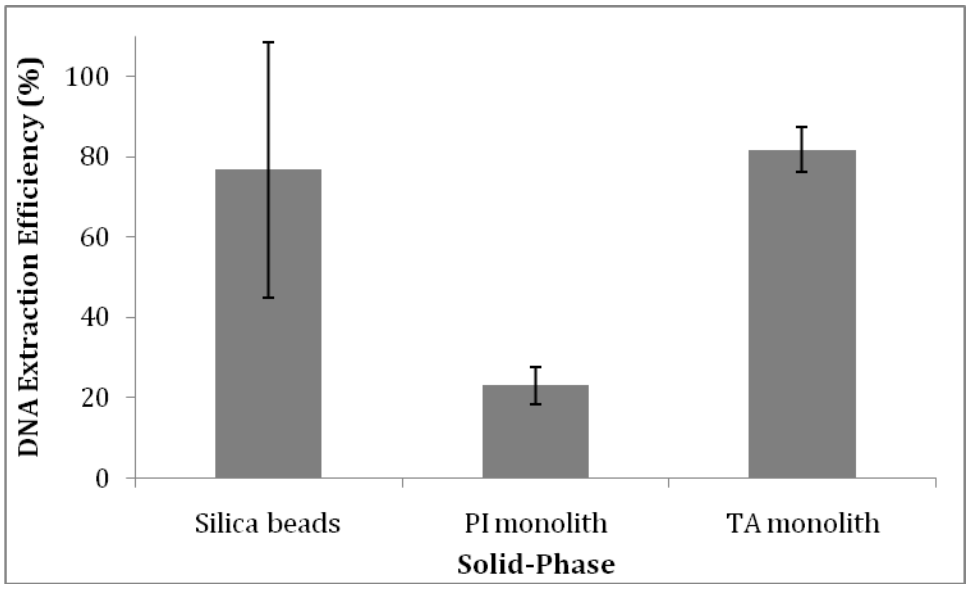


Figure 3.14: **Comparison of the different silica-based solid-phases for DNA extraction. The error bars represent the % standard deviation of the DNA extraction efficiencies (n≥4).**

In addition to the DNA extraction efficiency of the different solid-phases, it is also important to consider the practicalities associated with their production and precision placement. While the simplest method of producing the solid-phase is to simply add silica beads to the DNA extraction chamber, to produce an exact monolayer reproducibly is much more challenging, thus resulting in a large variation in the DNA extraction efficiencies obtained. In addition, a 24 hour incubation with TE buffer is required prior to the DNA extraction process, which is very time consuming, although once the silica beads have been treated they can be washed in distilled water and stored until required. Initial work using thermally activated monoliths revealed that while they showed high DNA extraction efficiency it was difficult to obtain accurate positioning within the DNA extraction chamber without leakage into the DNA amplification chamber occurring. This prompted the move to investigate the use of photo-initiated monoliths as their positioning is much easier to control using UV light by masking off those areas which do not require polymerisation, however, a low DNA extraction efficiency was found to be associated with these monoliths. It was therefore decided that the thermally activated monoliths would be the solid-phase of choice for inclusion in the microfluidic device due to ease of solid-phase production, accurate placement within the device, high DNA extraction efficiency and superior reproducibility.

3.3.7 DNA Extraction from Biological Samples

It is important for forensic applications that the DNA extraction methodology be able to cope with a range of biological samples that may be present at the scene of a crime. The focus of this work was to create a portable DNA analysis instrument which could analyse both scene of crime and CJ samples. For CJ samples, the DNA analysis instrument would be located within a custody suite of a police station and so would only be used with buccal swabs as the source of DNA. However, at the scene of a crime a wide range of biological sample types could be encountered.

A range of biological sample types, namely buccal swabs, whole blood and dried blood, were examined using the optimal DNA extraction methodologies described above for each of the different solid-phase matrices (see details in Chapters 3.3.3, 3.3.4 and 3.3.5). Sufficient DNA was shown to be released from each of the biological sample types by incubation with GuHCl, as this serves to not only bind the DNA to the solid-phase but also can lyse cells and inactivate nucleases.[24] When dealing with older, possibly degraded samples, as is the case with dried blood, the amount of DNA released could be increased by adding ProK to the chaotropic salt solution using the method described in Chapter 2.2.1.

DNA was successfully extracted from buccal swabs and whole blood using silica beads, thermally activated potassium silicate monoliths and photo-initiated MPTMS monoliths using the optimised protocols for each of the solid-phases as described in Chapter 3.3.3, 3.3.4 and 3.3.5. However, the amount of DNA recovered varied considerably depending upon the solid-phase matrix used and an example of the DNA yields for the different matrices from a buccal swab is shown in Figure 3.15. As expected from previous studies of DNA extraction efficiency, the thermally activated potassium silicate monolith consistently produced the greatest DNA yield during the elution phase irrelevant of which biological sample type was analysed.

The eluted DNA was also tested to ensure that the extraction process was capable of removing all potential inhibitors of PCR by performing DNA amplification of the Amelogenin locus. Eluted DNA samples, 2 µl aliquots, were added to 8 µl of PCR reagent mixture (see Table 2.1) and thermally cycled using a conventional PCR instrument according to the conditions given in Chapter 2.4.2. Successful PCR, as determined using slab-gel electrophoresis, was achieved from DNA extracted from both buccal swabs and whole blood, using all three solid-phase matrices. While cellular and proteinaceous debris, which could potentially inhibit PCR, were removed using an alcohol wash to a sufficient level for amplification to occur, significant problems with the blocking of pores in the silica bead-based system were observed. Such blockages resulted in high back

pressures which in severe cases led to the system becoming unusable and so DNA could not be eluted, again supporting the use of monoliths as the solid-phase in place of silica beads.

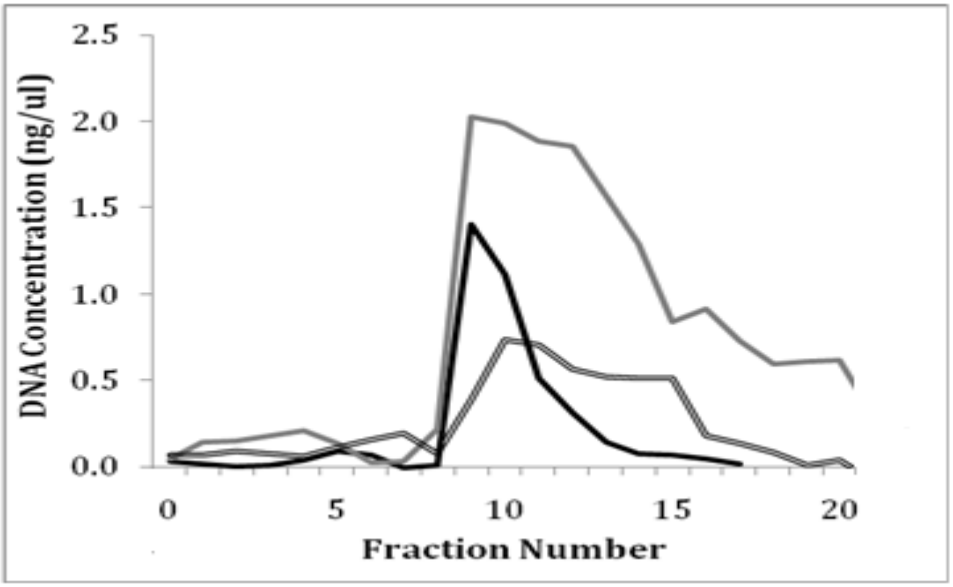


Figure 3.15: DNA extraction from buccal swabs using different solid-phases matrices. Buccal swabs incubated with GuHCl and the resulting solution added to the DNA extraction chamber containing either silica beads (——), thermally activated potassium silicate monolith (——) or photo-initiated MPTMS monolith (——).

While the collection of buccal swabs in a police custody suite would provide fresh biological samples for analysis, if the microfluidic system were to be taken to the scene of a crime then it is likely that the samples will not be fresh. Crime scenes are not necessarily discovered immediately and even then it can take time to collect biological samples for analysis. Therefore it was decided to test dried whole blood samples, exposed to air for up to 14 days, to more realistically replicate biological samples that are likely to be present at the scene of a crime. While DNA could be successfully extracted from 14 day old blood

samples using all three solid-phase matrices, the extraction efficiencies for the silica beads and photo-initiated MPTMS monoliths (17.2% and 7.5% respectively) were poor in comparison to the thermally activated potassium silicate monoliths (50.6%). Furthermore, when the eluted DNA was subjected to PCR amplification, only DNA extracted using the thermally activated potassium silicate monoliths successfully yielded PCR products.

3.3.8 Use of Carrier RNA in DNA Extraction Protocols

Obtaining the maximum amount of DNA from a biological sample is crucial to the success of any DNA extraction method. The use of carrier molecules has been reported to aid the recovery of DNA in macro-scale systems; for example the addition of a co-precipitant, glycogen, to phenol-chloroform extraction protocols increases the precipitation of DNA in the presence of alcohol.[52] The use of poly-A carrier RNA in commercially available Qiagen DNA extraction kits has also been reported, with an average increase in the DNA recovered during the elution phase of 24%, with more prominent effects when lower DNA concentrations were used.[53]

While the use of carrier molecules has been successfully applied to commercial DNA extraction protocols, their potential application in microfluidic systems has yet to be reported. In order to evaluate this, a modified version of the optimised DNA extraction protocol for thermally activated monoliths (see Chapter 3.3.4) was performed in which poly-A carrier RNA [Qiagen, UK] was included in the GuHCl binding solution. Preliminary work, carried out by BSc project supervisee Lauren Thain, demonstrated that the addition of poly-A carrier RNA to the GuHCl solution increased the DNA extraction efficiency on thermally activated potassium silicate monoliths in 0.6 mm i.d. glass capillaries [Blaubrand® intraMARK, Germany]. Therefore an evaluation as to the extent to which the poly-A carrier RNA could improve DNA recovery was undertaken. Optimisation of the

amount of carrier RNA required for maximum DNA extraction efficiency showed that a ratio of carrier RNA to DNA of between 10:1 and 50:1 resulted in the highest DNA extraction efficiency (Figure 3.16). (N.B. These experiments were carried out in glass capillaries rather than the microfluidic devices and prior to optimisation of the TE buffer pre-treatment step. As a result the DNA extraction efficiencies are lower than that of the optimised system demonstrated in Chapter 3.3.5 but the overall trend is clearly apparent).

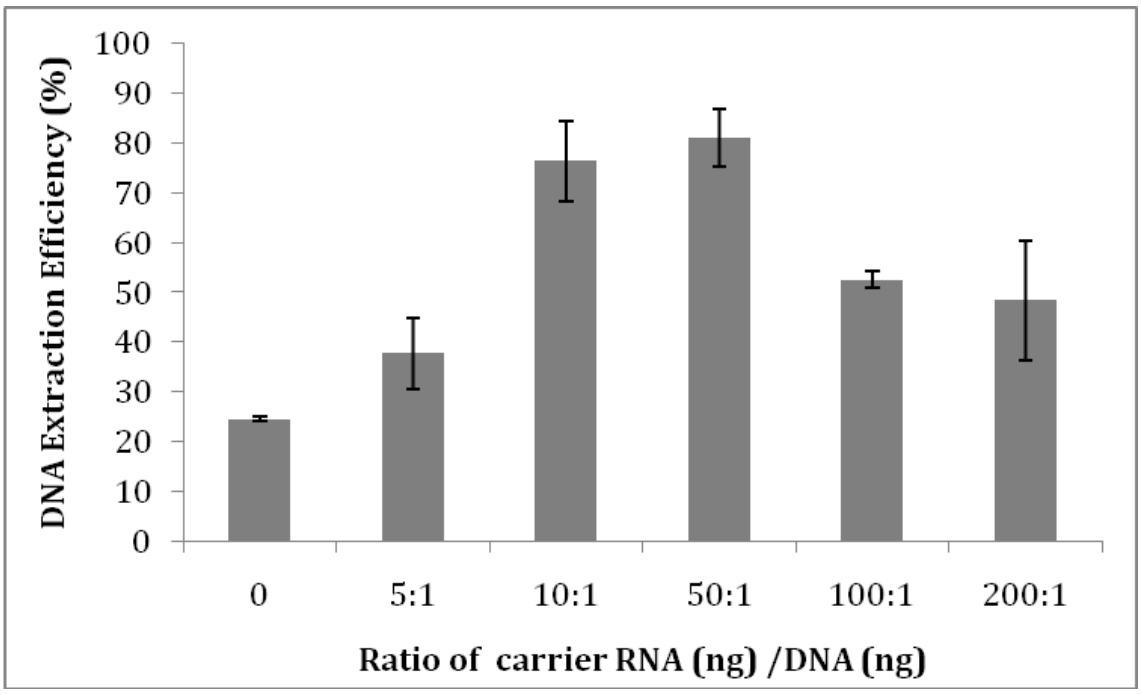


Figure 3.16: Effect of the ratio of carrier RNA (ng) to DNA (ng) on DNA extraction efficiency. Poly-A carrier RNA was added to DNA in a GuHCl binding solution prior to DNA extraction being performed on thermally activated monoliths in a microfluidic environment (n=3).

In commercial DNA extraction systems, the use of carrier RNA has been shown to have a pronounced effect when the amount of DNA present in the binding solution was limited.[53] The use of poly-A carrier RNA was also shown to have a more prominent effect at higher DNA concentrations when DNA extraction was performed in the microfluidic system (Figure 3.17).

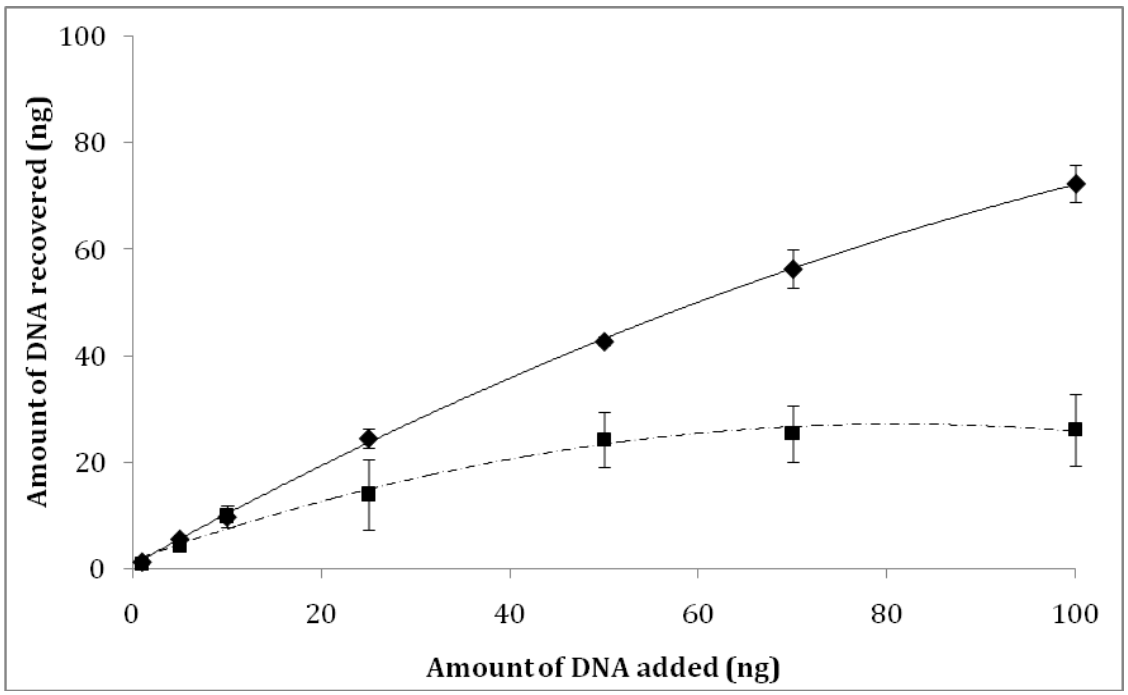


Figure 3.17: Effect of the amount of DNA recovered from the thermally activated potassium silicate monolith during the elution step compared with the amount of DNA initially added. Samples with poly-A carrier RNA at a ratio of 10:1 (◆) were compared to those with no carrier RNA (■) added to the DNA/GuHCl binding solution (n=3).

It is hypothesised that on a silica solid-phase matrix there are always a certain number of sites which will irreversibly bind nucleic acids. Poeckh *et al*., studied the adsorption isotherm behavior of DNA and RNA to silica in the presence of guanidine hydrochloride and showed that RNA adsorption was more efficient [54]. Therefore by including carrier RNA in the binding solution it can sacrificially bind to these sites and so the loss of important DNA is minimised leading to greater recoveries during the elution phase.[53]

PCR analysis was carried out on the eluted DNA not only to confirm the integrity of the DNA but also to establish that the carrier RNA had no adverse effects on downstream applications. Eluted DNA samples, 2 µl aliquots, were added to 8 µl of PCR reagent mixture (see Table 2.1) and thermally cycled using a conventional PCR instrument

according to the conditions given in Chapter 2.4.2. The results, following slab-gel electrophoresis, showed successful amplification of the target TH01 locus indicating that the extracted DNA is of sufficient quality for amplification and furthermore that the carrier RNA does not inhibit the reaction (Figure 3.18).

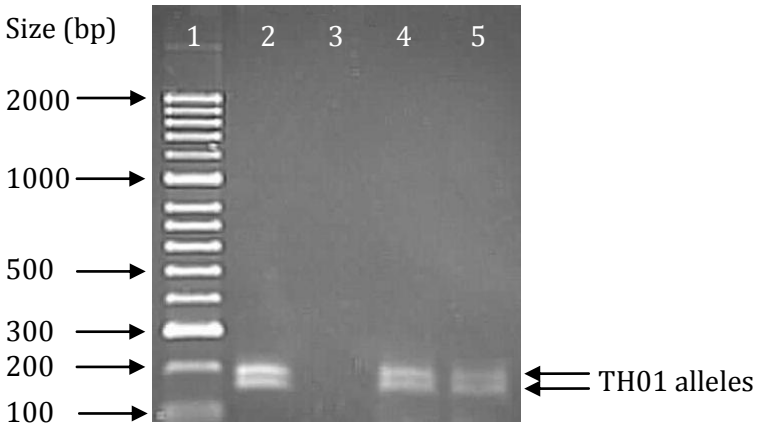


Figure 3.18: Effect of carrier RNA on PCR amplification of eluted DNA. UV transilluminator image of slab-gel electrophoresis results showing the effects of carrier RNA on DNA amplification. DNA, with or without carrier RNA, in GuHCl binding solution was extracted on a thermally activated silica monolith. Lane 1: DNA size ladder; Lane 2: positive control using genomic DNA extracted with QIAamp® DNA Micro Kit; Lane 3: negative control containing no DNA; Lane 4: amplified DNA extracted using silica monolith with carrier RNA; Lane 5: amplified DNA extracted using silica monolith with no carrier RNA.

3.3.9 Amplification of Extracted DNA

All the results described so far have revolved around quantitation of the DNA in order to allow the efficiency of the process to be calculated and therefore optimised. Whilst it is important to have an understanding of the DNA extraction efficiency, ultimately the eluted DNA needs to be compatible with PCR amplification. There are many substances which have the ability to inhibit PCR, for example haemoglobin found in whole blood samples,

and therefore require removal during the DNA extraction process in order to facilitate successful amplification of the DNA.

Sequentially collected 2 µl aliquots of DNA eluted from each of the three types of solid-phase matrix were added to 8 µl of PCR reagent mixture (see Table 2.1) and thermally cycled using a conventional PCR instrument according to the conditions given in Chapter 2.4.2. The results, following slab-gel electrophoresis, showed successful amplification of the target D8 S1179 and D16 S539 loci. An example of the results obtained when silica beads were used as the solid-phase is shown in Figure 3.19, where duplex PCR products from sequentially eluted fractions are visible.

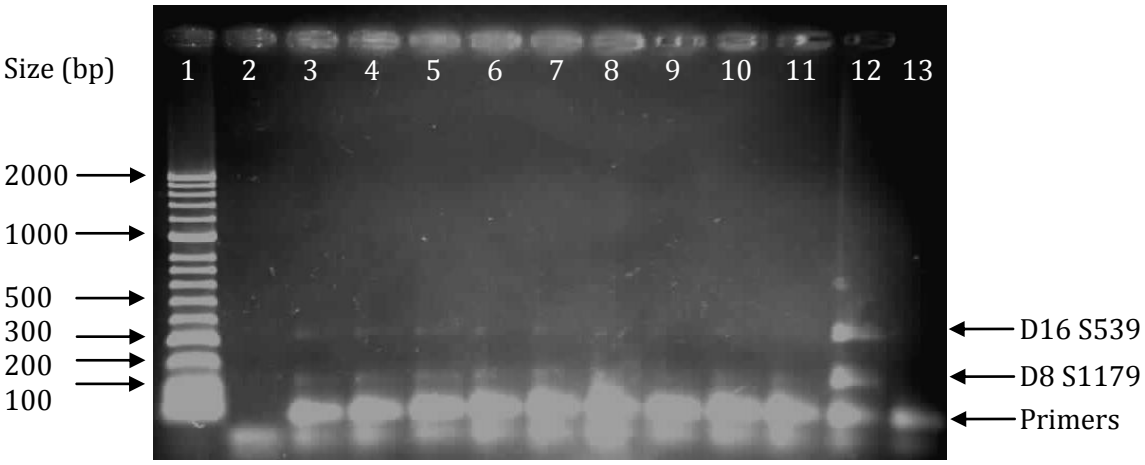


Figure 3.19: PCR amplification of DNA extracted using the microfluidic system. UV transilluminator image of slab-gel electrophoresis results showing the successful amplification of DNA eluted from silica beads. Lane 1: DNA size ladder; Lanes 2-11: sequentially eluted DNA fractions from the microfluidic device; Lane 12: positive control and Lane 13: negative control.

It is noteworthy that the first eluted DNA fraction (Lane 2) does not show any PCR products, which is most likely due to the presence of residual isopropanol from the wash step. Alcohols, such as isopropanol and ethanol, are commonly used during DNA extraction to remove proteins and cellular debris which can affect downstream

applications, however the alcohols themselves are known inhibitors of PCR if present in too high a concentration. One of the main reasons for this inhibition is a loosening of the DNA polymerase conformation due to a decrease in the hydrophobic effect.[204]

As there may be a limited amount of sample available it is important that as much of the eluted DNA as possible is suitable for PCR amplification. By studying a typical DNA elution profile, for example that seen in Figure 3.9, it is clear that the majority of the DNA is eluted in the initial fractions. Therefore it is likely that while the first fractions eluted do not support successful DNA amplification they are actually the ones that contain the most DNA. In light of this a number of techniques were investigated in an attempt to reduce or eliminate the problem of isopropanol carry over into the eluted fractions. The first method investigated was to air dry the solid-phase matrix after the wash step to remove any residual isopropanol before the DNA elution step was carried out. This technique resulted in successful amplification of the initial eluted fraction containing DNA (Figure 3.20).

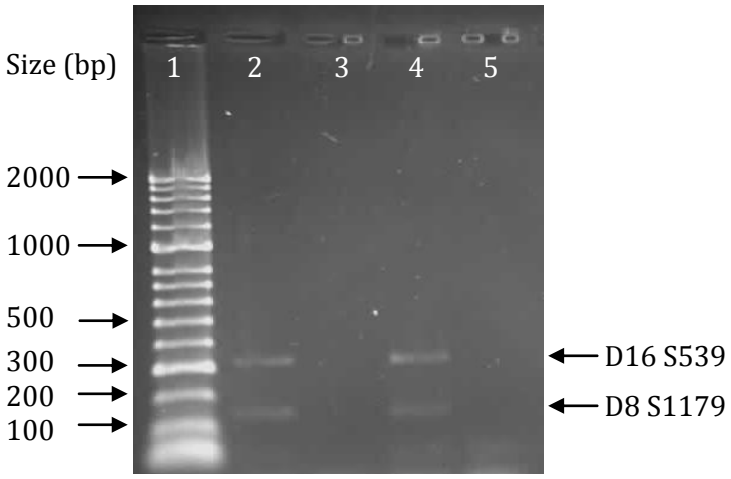


Figure 3.20: Effect of isopropanol evaporation on DNA amplification. UV transilluminator image of slab-gel electrophoresis results of isopropanol evaporation experiment. Lane 1: DNA size ladder; Lane 2: isopropanol evaporated from sample prior to elution step; Lane 3: isopropanol not evaporated from sample prior to elution step; Lane 4: positive control and Lane 5: negative no DNA control.

While the use of a drying step achieves the desired goal as demonstrated here, is not a practical step when carrying out SPE in a microfluidic device, mainly due to the fact that the chance of introducing bubbles into the system when the DNA is eluted is very high. Bubbles can cause problems not only with downstream applications such as PCR but also with the integration of multi-step processes on the same microfluidic device.

An alternative method to reduce the amount of residual alcohol present in the initial eluted DNA fractions was therefore required and so the use of a gradient elution was evaluated. In this case the flow-rate of the isopropanol wash was gradually decreased whilst the flow-rate of TE buffer for elution is gradually increased, thus slowly reducing the percentage alcohol. Using isopropanol to 10 mM TE buffer flow-rates of 4:1, 3:2, 2:3, 1:4 and 0:5 µl/min sequentially. Despite a small amount of DNA being lost during the period of isopropanol dilution, the majority of the DNA eluted with a similar profile to those reported earlier without using a gradient (Figure 3.21). The eluted samples were then subjected to DNA amplification which showed that amplified loci were detectable from Lane 25, which corresponds to the first elution fraction and that which contains the maximum amount of DNA.

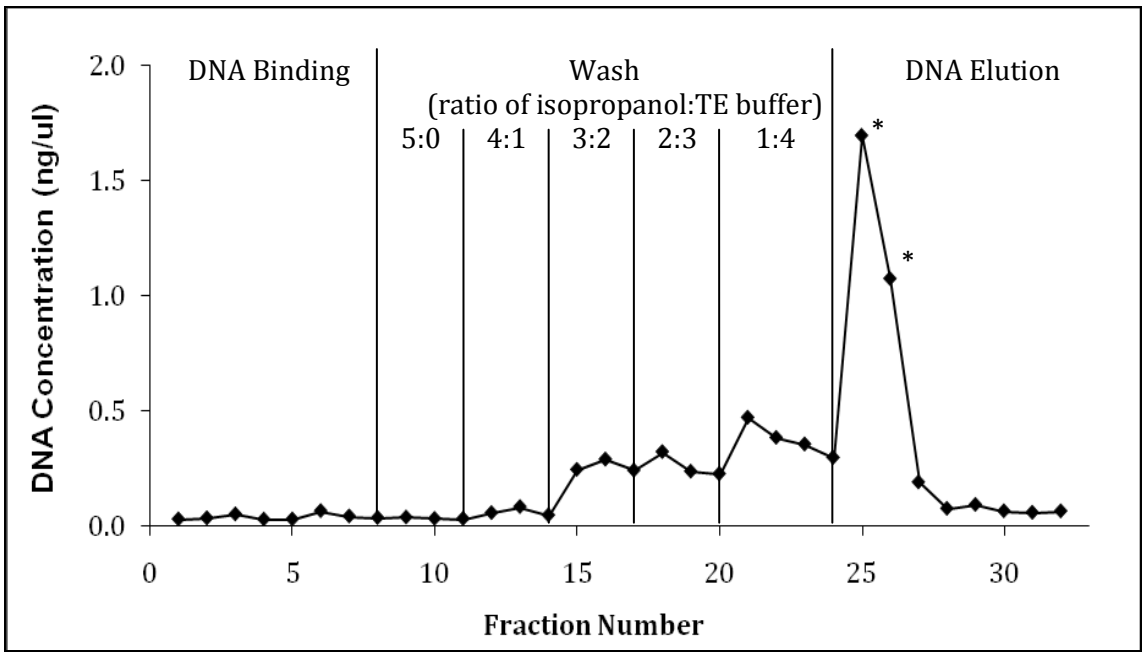


Figure 3.21: Effect of gradient elution on DNA elution profile and subsequent PCR amplification. Fractions marked with * indicate those which successfully produced PCR products after undergoing DNA amplification.

3.3.10 DNA Elution using PCR reagents

Current microfluidic systems which combine DNA extraction and amplification through hydrodynamic movement of the eluted DNA between chambers rely on the use of low ionic strength buffers for elution.[191, 192] As DNA amplification is performed in the presence of a low ionic strength buffer it was hypothesized that DNA elution could be carried out using a 'master mix' solution which contains all the necessary PCR reagents which would allow easier integration of the DNA extraction and amplification steps.

Initial studies were carried out using a cylindrical DNA extraction chamber, 0.6 mm diameter by 3mm height, facilitating multi-layer silica bead packing for the solid-phase. DNA was extracted from saliva using the optimised protocol given at the end of Chapter 3.3.3, with elution carried out using a standard PCR reagent mixture (see Table 2.1), rather than 10 mM TE buffer. Sequentially eluted 10 µl fractions were then thermally cycled

using a conventional PCR instrument according to the conditions given in Chapter 2.4.2. Successful multiplex amplification, of D8 S1179 and D16 S539 loci, from sequentially eluted DNA-containing fractions was observed as indicated by the presence of PCR products, following slab-gel electrophoresis (Figure 3.22).

Figure 3.22: PCR amplification of DNA eluted from the microfluidic DNA extraction system using PCR reagents. UV transilluminator image of slab-gel electrophoresis results from DNA eluted in PCR reagents. Lane 1: DNA size ladder; Lanes 2-9: sequentially eluted DNA fractions; Lane 10: positive control and Lane 11: negative no DNA control.

However, when the surface area to volume ratio of the DNA extraction chamber was increased i.e. when a silica bead monolayer or monoliths were used, then elution of DNA from the solid-phase using PCR reagents failed to produce PCR products after amplification. It was hypothesised that at these higher surface area to volume ratios PCR inhibition was occuring due to adsorption of the DNA polymerase to the surface. By spiking the eluted DNA samples with additional DNA polymerase immediately prior to DNA amplification positive results were obtained thereby confirming the hypothesis. While a multitude of techniques exist by which the surface of the microfluidic device can be treated to prevent adsorption of the DNA polymerase (see Chapter 4.2.2 for some examples) these treatments were found to be removed by the GuHCl and alcohol solutions

required for the DNA extraction process. In addition modification of the surface in such a way would also affect the ability of the DNA itself to be adsorbed during the DNA binding step. It was therefore concluded that while the elution of DNA with PCR reagents is an attractive option in developing an integrated DNA extraction and amplification device, its use is restricted to DNA extraction chambers of low surface area to volume ratio. As with most integrated systems, a high degree of compromise between the individual components is required. Therefore the advantages of eluting with PCR reagents must be weighed against having a reduced surface area to volume ratio for the DNA extraction chamber, which decreases the surface area available for DNA adsorption and therefore efficiency of the extraction process, and limitations resulting from the wet etching technique used for microfluidic device manufacture which means all integrated features must be etched to the same depth.

3.4 Summary

The use of SPE methodology enables not only purification but also pre-concentration of DNA from biological samples. A range of silica-based solid-phase matrices have been investigated for their potential inclusion in an integrated microfluidic device for performing DNA analysis. The use of silica as the solid-phase enables the elution of DNA in a PCR compatible format, which offers considerable advantage over ion-exchange methods which require further processing of the eluted DNA due to the high alkalinity of the elution buffer solutions used.[79]

The choice of silica-based solid-phase is important as it has a substantial effect on the efficiency of the DNA extraction process. Despite initial successes using a packed bed of silica beads for DNA extraction, efficiency studies showed a significant lack of reproducibility. Besides this, the non-uniform packing of the silica beads often led to problems with high back-pressures caused by blocking of the pores with cellular debris. This prompted a move to silica-based monoliths which, due to a higher degree of control in production, exhibit much greater reproducibility. Those monoliths produced by thermal activation showed higher DNA extraction efficiency and were easier to produce than photo-initiated monoliths.

The increased surface area available for DNA adsorption along with the decreased diffusional distance (Equation 1.2) created using a solid-phase matrix has a significant effect on DNA extraction efficiency. The diffusional distance (D) for DNA is related to length (L) in base pairs where:[205]

$$D = L^{-0.6} \qquad \textbf{(Equation 3.4)}$$

The length of the DNA subjected to DNA extraction on the microfluidic device cannot be known with certainty and therefore calculation of diffusional distance is difficult as large variations will result if the sizing is not accurate.

Under optimised conditions, using a thermally activated monolith, (Figure 3.14) DNA extraction efficiencies using the microfluidic device were 82 ± 6%. Such DNA extraction efficiencies are equivalent to those reported in the literature for other monoliths on microfluidic devices but with a much simpler preparation procedure which would be more amenable to the mass production of microfluidic devices for commercialisation of an integrated genetic analyser (Table 3.1).

Method	Efficiency
K_2SiO_3 and formamide mixed, injected into chamber and placed in oven at 90°C overnight.	≤82%
TEOS in water hydrolysed by addition of nitric acid and heating to 60 °C for 10 minutes and then 80 °C for 60 minutes with stirring at 200 rpm. Channel filled with silica beads and TEOS solution placed in oven at 50 °C, heated to 300 °C at 8°C/minute, and held at temperature for 3 hours.[67, 68]	≤70.6%
TMOS, poly (ethylene glycol) and acetic acid stirred at 0°C for 45 minutes. The solution was then injected into a channel, the filled chip covered and placed in an oven at 40°C for 10 hours to gel.[72, 73]	≤85%
A monomer solution of TMSPM and HCl was stirred at room temperature in the dark for 20 minutes. A sol-gel solution was prepared with monomer solution, toluene and photoinitiator (Irgacure 1800) and stirred in the dark at room temperature for 5 minutes. The filled capillary then exposed to UV light prior to an ethanol wash to remove excess monomer reagent. The monolith was then derivatised using TMOS for 45 minutes.[75-77]	≤85%

Table 3.1: Comparison of production protocols for DNA extraction monoliths reported in the literature, showing the ease of production of the thermally activated monolith reported in this chapter. Efficiency corresponds to the DNA extraction efficiencies for simple λ-phage DNA.

In comparison to commercially available DNA extraction kits, in this example a Qiagen™ DNA extraction kit, the extraction efficiency is much higher using the microfluidic system, 82% compared to 49%.[75] Additionally the number of steps required for performing the analysis is reduced to three steps: DNA loading, wash and DNA elution in the microfluidic system. In the commercial Qiagen™ DNA extraction kit, 15 individual steps are required including many centrifuging stages. By eliminating such steps the simplicity of the process is increased and this in turn reduces the amount of time taken, from around 2 hours in conventional systems to 20 minutes in the microfluidic system presented here.

Using the optimal DNA extraction conditions described above for thermally activated monoliths in the microfluidic device, DNA of PCR amplifiable quality was extracted from buccal cells, whole and dried blood samples. In addition the effects of carrier RNA on performing DNA extraction in a microfluidic environment have been reported for the first time. The inclusion of carrier RNA to the DNA binding solution was shown to increase DNA extraction efficiency with no adverse effects on the downstream PCR amplification reaction.

The potential for the optimised DNA extraction method presented here to be incorporated in an integrated microfluidic device for performing DNA extraction and amplification will be discussed in detail in Chapter 5. Apart from the purity and quantity of the extracted DNA, it was found that the presence of residual isopropanol from the wash step plays a definitive role in determining whether or not DNA amplification will be successful. While isopropanol is a known inhibitor of PCR, the use of a gradient elution for desorbing DNA from the silica overcomes this potential problem.

4 DNA Amplification on a Microfluidic Device

4.1 Introduction

This chapter aims to investigate multiplex DNA amplification on a microfluidic device. While a variety of isothermal DNA amplification techniques exist, they are mainly used for amplification of RNA or circular genomes,[90] and so it is the use of the polymerase chain reaction which will be looked at in detail here. In order to enable the production of a DNA profile for forensic analysis purposes, multiplex PCR is essential to provide a high degree of discrimination between individuals. DNA amplification is required to enable easier detection of the specific loci of interest. As well as increasing the number of copies of the alleles present at each locus, the incorporation of labelled primers can be tailored for specific detection techniques. In this study, fluorescent tags will be utilised as this replicates current commercially available forensic DNA profiling kits and fluorescent detection can be readily applied to microfluidic devices.

A stationary PCR chamber was chosen, in preference to a flow-through chamber design, in which the PCR reagents are contained within a single chamber and cycled through the required temperatures. As flow-through designs pass the PCR reagents through distinct temperature zones, resulting in rapid temperature transitions, continuous sample volumes are produced. However, the application here requires PCR amplification of discrete samples for forensic purposes. The use of stationary PCR maintains flexibility, in terms of cycle number, which is important in being able to optimise developmental systems. While stationary PCR chambers have been more widely integrated with downstream processes, such as capillary electrophoresis, recent developments in droplet-based microfluidics have shown that two-dimensional separations are possible using droplet manipulation techniques and could be used following DNA amplification to enable analysis of individual droplets.[206]

In stationary PCR the speed of the reaction is limited by the ramping rate of the heater but good fluidic and temperature control is provided which is important in producing an optimised DNA amplification protocol. Contact heating methods enable precise temperature control but can suffer from slow temperature ramp rates. Non-contact heating methods can provide faster ramping rates but temperature control can be more difficult. Therefore examples of both contact and non-contact heating methods were evaluated for use in the miniaturised genetic analysis system, namely Peltier and microwave heating.

In the previous chapter on DNA extraction, the increased surface area to volume ratio present in the microfluidic system proved advantageous. In fact, it was important to increase the surface area even further, through the incorporation of a solid-phase matrix, to achieve maximum DNA adsorption and therefore DNA extraction efficiency. In contrast, the increased surface area is problematic when performing DNA amplification as DNA polymerase adsorption occurs.[122] Here, a variety of surface coating techniques are evaluated, in combination with dynamic passivation methods, to provide a minimal interaction of the PCR reagents with the internal surfaces of the microfluidic device.

In addition to end-point analysis, using conventional gel electrophoresis techniques, real-time monitoring of PCR was investigated as a means of performing DNA quantification on the microfluidic device. DNA amplification was monitored using both intercalating dye and TaqMan™-style probe technologies.

4.2 Experimental

4.2.1 Microfluidic Device Design for DNA Amplification

Microfluidic devices were manufactured by Dr. Steve Clark at the University of Hull, according to the protocol given in Chapter 2.1. Two different microfluidic device designs were used for PCR depending upon the heating method to be used. DNA extraction and amplification could both be performed on the microfluidic device shown in Figure 4.1a, using the Peltier heating system. In addition, a second microfluidic device was designed for use in the microwave heating cavity, containing only a DNA amplification chamber (Figure 4.1b). Various etch depths (ranging from 300 µm to 100 µm) were used giving rise to chambers with volumes ranging from 2.1 µl to 0.7 µl.

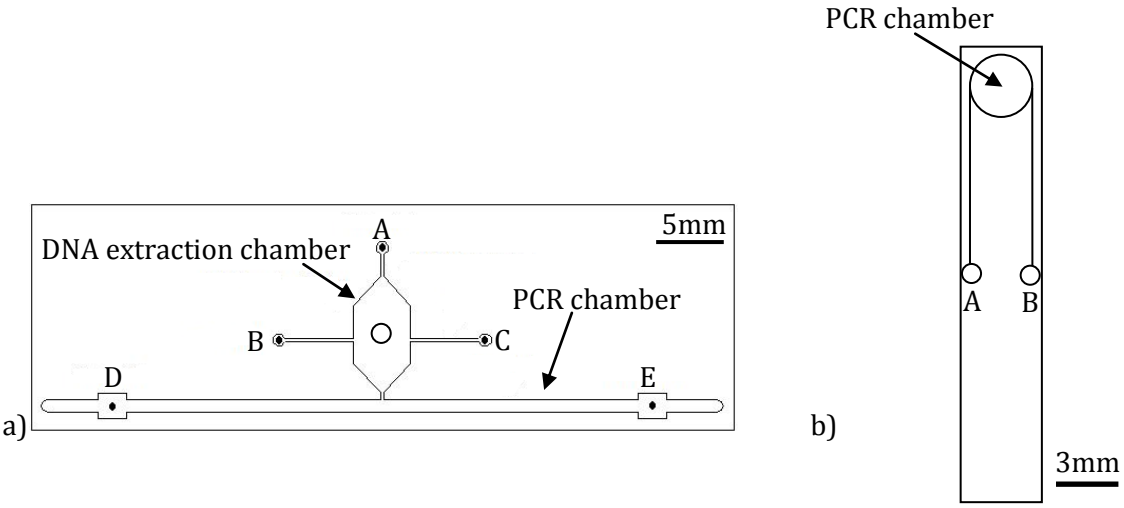


Figure 4.1: Microfluidic device design. a) Schematic showing the design of the full microfluidic device for combined DNA extraction and amplification, including 360 µm ports (A-E) for addition of PEEK tubing. A 1 mm port was included in the centre of the DNA extraction chamber to facilitate addition of the solid-phase matrix into the microfluidic device. All features are etched into the top plate to a depth of 100 µm; b) Schematic showing the design of the microfluidic device for DNA amplification using microwave heating cavity, including 360 µm ports A and B for attachment of PEEK tubing.

4.2.2 Surface Passivation Techniques

Dynamic passivation of the internal glass surfaces of the DNA amplification chamber was achieved using 0.2 µg/µl BSA [New England Biolabs, UK], with the optional addition of 0.01% (w/v) PVP [Sigma-Aldrich, UK] and 0.1% (v/v) Tween-20 [Sigma-Aldrich, UK] to the PCR reagent mixture.[167]

Alongside the dynamic passivation, a number of silanisation methods were investigated for their compatibility with PCR and ability to prevent DNA polymerase adsorption to the internal glass surfaces of the microfluidic device. An overview of the different methods evaluated is given in Table 4.1. Prior to silanisation, the microfluidic device was cleaned using sequential washes of 1 M sodium hydroxide, purified water and ethanol at 5 µl/min for 30 minutes each before being placed in an oven at 90 °C to dry.

Technique	Silanisation Solution	Flow Rate	Time
OTS	A 10 µl aliquot of octadecyltrichlorosilane (OTS) [Sigma-Aldrich, UK] was added to 2 ml chloroform [Fluka, UK] and 8 ml hexadecane [Sigma-Aldrich, UK]. Adapted from [207]	1 µl/min	Overnight
SafetyCoat™	SafetyCoat™ [JT Baker, US].[104]	5 µl/min	5 minutes
SigmaCote™	SigmaCote™ [Sigma-Aldrich, UK].[120]	5 µl/min	5 minutes
TPS	A 290 µl aliquot of trichloro((1*H*,1*H*,2*H*,2*H*) perfluorooctyl)silane (TPS) in 5 ml 2,2,4-trimethyl pentane.[208]	5 µl/min	10 minutes

Table 4.1: An overview of the silanisation techniques evaluated and how they were applied to the microfluidic device.

After each passivation treatment, the microfluidic device was washed sequentially at 5 µl/min for 5 minutes with acetone and purified water to remove any unreacted or waste products.

Silanisation of the glass changes the surface properties from hydrophilic to hydrophobic therefore the success of the process can be measured by determining the contact angle of a droplet of water with the internal glass surfaces. This was done by obtaining a microscope image of the microfluidic channel and measuring the water contact angle using ImageJ software (Figure 4.2).

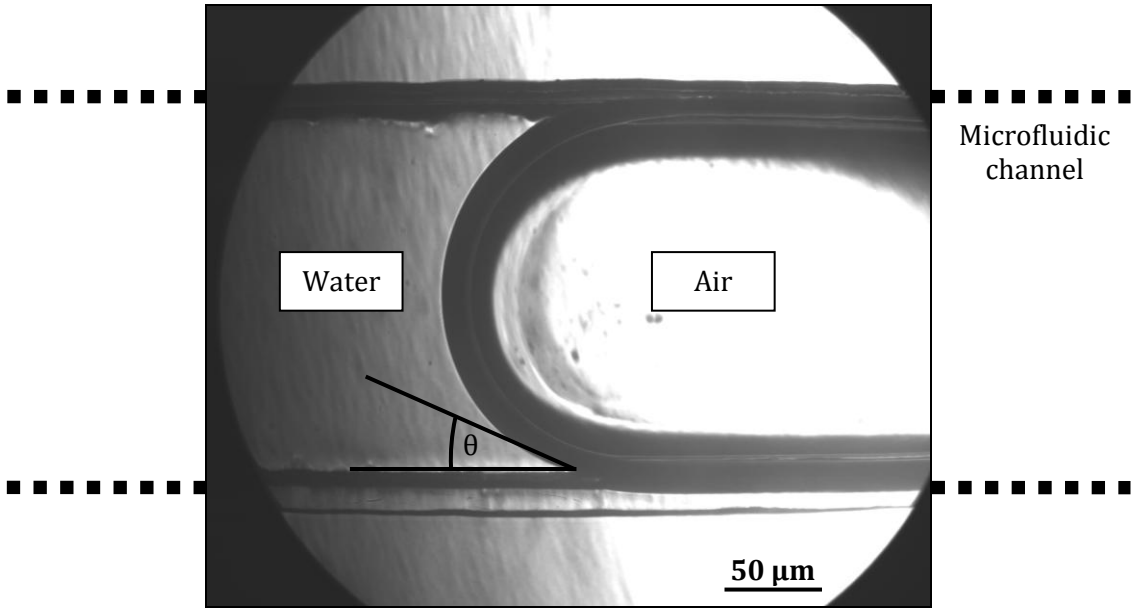


Figure 4.2: Contact angle measurement within a microfluidic device. Example showing how the contact angle (θ) was measured in the microfluidic channel using microscopy and ImageJ software.

4.2.3 DNA Amplification using Hydrodynamic Pumping

PCR reagents for amplification of an individual locus or custom-made multiplex reaction were prepared, as described in Table 2.1, made up to the desired volume in purified water. Extracted human genomic DNA (either using a QIAamp® DNA Micro Kits [Qiagen, UK] or the microfluidic system described in Chapter 3) was quantified using PicoGreen™ and added at a concentration of 1 ng to 50 µl of PCR reagents.

When using the AmpF*l*STR® Second Generation Multiplex (SGM) Plus™ kit [Applied Biosystems, UK] the manufacturer's protocol was followed. This involved mixing 21 µl of AmpF*l*STR® PCR Reaction Mix, 11 µl of AmpF*l*STR® SGM Plus™ Primer Set and 1 µl of AmpliTaq Gold® DNA polymerase. A 30 µl aliquot of this PCR master mix was then added to 20 µl of AmpF*l*STR® Control DNA 007. The thermal cycling parameters required for DNA amplification using the SGM Plus™ kit are:

Initial incubation:	95°C for 11 minutes	
DNA denaturation:	94°C for 1 minute	28 cycles
Primer annealing:	59°C for 1 minute	
DNA extension:	72°C for 1 minute	
Final extension:	60°C for 45 minutes	

An aliquot of either PCR reagent mixture was then added to the microfluidic device using a standard displacement pipette through port D of the microfluidic device shown in Figure 4.1a or port A of the microfluidic device shown in Figure 4.1b. It was found in preliminary experiments that the PEEK tubing and MicroTight® Adapters previously used for the DNA extraction work (Chapter 3) were, in part, responsible for the adsorption of the DNA polymerase resulting in inhibition of PCR. This adsorption was not be prevented by any of the surface passivation treatments described in Chapter 4.2.2, therefore manual sample injection was used in place of standard hydrodynamic pumping.

For each experiment, positive and negative controls and 'push-through' samples were ran on a standard TC-312 thermal cycler. 'Push-through' samples were generated when the PCR reagent mixture was flowed through the microfluidic device and collected from the outlet, without any thermal cycling on the microfluidic device. This enabled the surface passivation to be tested, since if this sample was not successfully amplified after removal from the device it suggested that the DNA polymerase had been adsorbed during flow through the device preventing it from participating in the amplification reaction.

4.3 Results & Discussion

4.3.1 Optimisation of Single Locus PCR in a Conventional Thermal Cycler

Optimisation of the amplification reaction required for performing single locus PCR was necessary for each of the primers pairs used: Amelogenin, D3 S1358, D8 S1179, D16 S539, D18 S51, D21 S11, FGA, TH01 and vWA (see Table 2.3 for detailed primer information). The annealing temperature (range of 56-62°C) and primer concentrations (range of 0.1-0.5 µM) were varied (Figure 4.3). When investigating annealing temperature, a constant primer concentration of 0.5 µM was used and when varying primer concentration, an annealing temperature of 60°C was used.

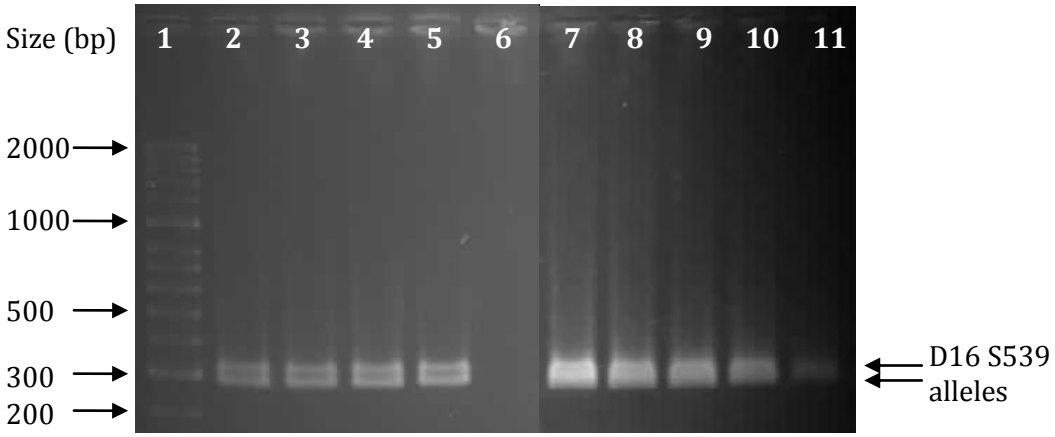


Figure 4.3: Single locus DNA amplification optimization. UV transilluminator image showing slab-gel electrophoresis photograph showing an example of the optimisation results from a single locus, in this case D16 S539, using both a range of annealing temperatures and a range of primer concentrations. Lane 1: DNA size ladder; Lanes 2-6: annealing temperature optimisation (56°C; 58°C; 60°C; 62°C; negative no DNA control); Lanes 7-11: primer concentration optimisation (0.5 µM; 0.4 µM; 0.3 µM; 0.2 µM; 0.1 µM).

A summary of all the results obtained from optimisation of annealing temperatures and primer concentration is given in Table 4.2. While the optimal conditions for each individual reaction have been determined, there are a wide range of conditions over which the primer sets function which is important when developing a multiplex reaction.

	Temperature (°C)				**Primer Concentration (µM)**				
Locus	56	58	60	62	0.1	0.2	0.3	0.4	0.5
Amelogenin	*	**	***	**	***	**	**	*	*
D3 S1358	**	**	***	**	***	**	**	*	*
D8 S1179	**	**	***	**	*	**	**	***	*
D16 S539	*	*	**	***	***	**	**	*	*
D18 S51	***	**	*	*	**	**	***	**	*
D21 S11	**	**	***	**	*	*	**	***	*
FGA	*	*	**	***	***	**	**	**	*
TH01	*	*	**	***	***	**	**	*	*
vWA	***	**	**	**	*	*	**	***	**

Table 4.2: Qualitative analysis of PCR products from all 9 forensically relevant loci as analysed by slab-gel electrophoresis, where * is the optimum, ** represents a strong signal and * a relatively weak signal.**

4.2.3 Surface Area Effects on DNA Amplification

The high surface area to volume ratio present in microfluidic systems, as compared to conventional PCR tubes, has been shown to have a dramatic effect on the ability to perform DNA amplification. Erill *et al.* carried out a systematic analysis of the interaction between glass-silica surfaces and PCR reagents and found that at high surface area to volume ratios DNA polymerase adsorption occurs resulting in inhibition of PCR.[122] Glass

tubes which mimicked the dimensions of a conventional 0.2 ml polypropylene PCR tube were produced in house by the University of Hull glassblower. Performing DNA amplification in both tube types using the Peltier heating system showed that there was no difference in PCR efficiency. Having kept the surface area to volume ratio the same and varying the material used this confirms that PCR is not inhibited by the material alone.

In order to further investigate surface area to volume ratio effects glass capillaries [Blaubrand® intraMARK, Germany], with varying internal diameters, were used. PCR was performed using the bench-top Peltier heating system for 28 cycles in the glass capillaries and the samples analysed using slab-gel electrophoresis to see if the DNA amplification reactions were successful. Inhibition of PCR was found to occur at surface area to volume ratios greater than 10:1 (Table 4.3).

Surface Area (mm^2)	Volume (µl)	Ratio	PCR
158.95	25	6:1	Yes
138.50	20	7:1	Yes
98.16	10	10:1	Yes
68.48	5	14:1	No

Table 4.3: **Analysis of the effect of surface area to volume ratio (given as mm^2:µl) of glass capillaries on the inhibition of DNA amplification.**

Analysis of the surface area to volume ratio of the microfluidic devices used here for DNA amplification (Figures 4.1a. 4.1b and 5.1) are shown in Figure 4.4. Using an etch depth of 300 µm results in a surface area to volume ratio that does not exhibit PCR inhibition but requires a relatively large volume. Such volumes do not offer any considerable advantage over conventional systems in terms of cost of analysis and also will suffer from slower

thermal transition therefore an etch depth of 100 µm is more appropriate. However, at this depth the surface area to volume ratio results in PCR inhibition and therefore surface passivation was required to overcome this.

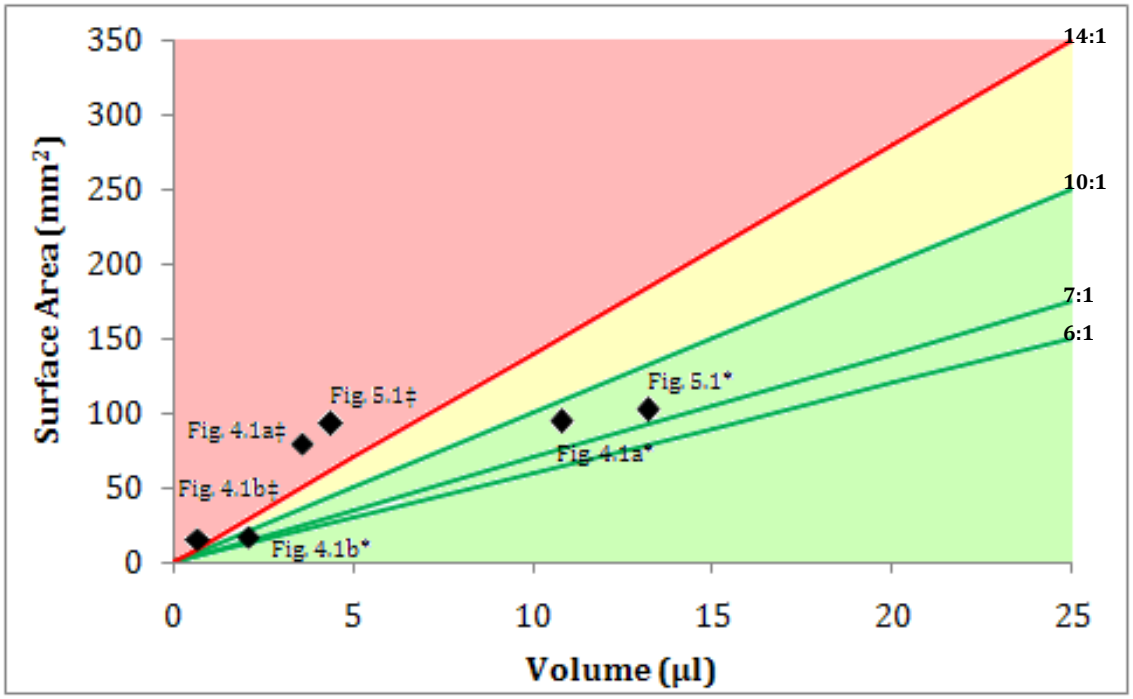


Figure 4.4: Graph depicting the success (green) or failure (red) of DNA amplification when performing PCR at different surface area to volume ratios based on the capillary results in Table 4.3. The surface area and volume details for the different microfluidic systems tested here are shown (◆) with etch depths of 100 µm (‡) and 300 µm (*), respectively.

Dynamic passivation was achieved using a previously reported combination of 0.2 µg/µl BSA, 0.01% (w/v) PVP and 0.1% (v/v) Tween-20.[167] While the use of dynamic passivation agents, such as BSA, results in competitive adsorption to the surface in place of the DNA polymerase some enzyme will still be adsorbed due to dynamic equilibrium. Whilst increasing the concentration of BSA can further reduce the effects of PCR inhibition, there

is a certain concentration threshold (≥ 5 μg/μl) above which adverse effects on the DNA amplification process occur due to an increased viscosity.[122]

A wide number of static passivation techniques have been reported in the literature for a variety of applications and a number have been evaluated here for use in the microfluidic DNA amplification chamber. As silanisation produces a more hydrophobic surface, the change in contact angle of water with the internal glass surface was used to assess the efficiency of each of these techniques (Figure 4.5).

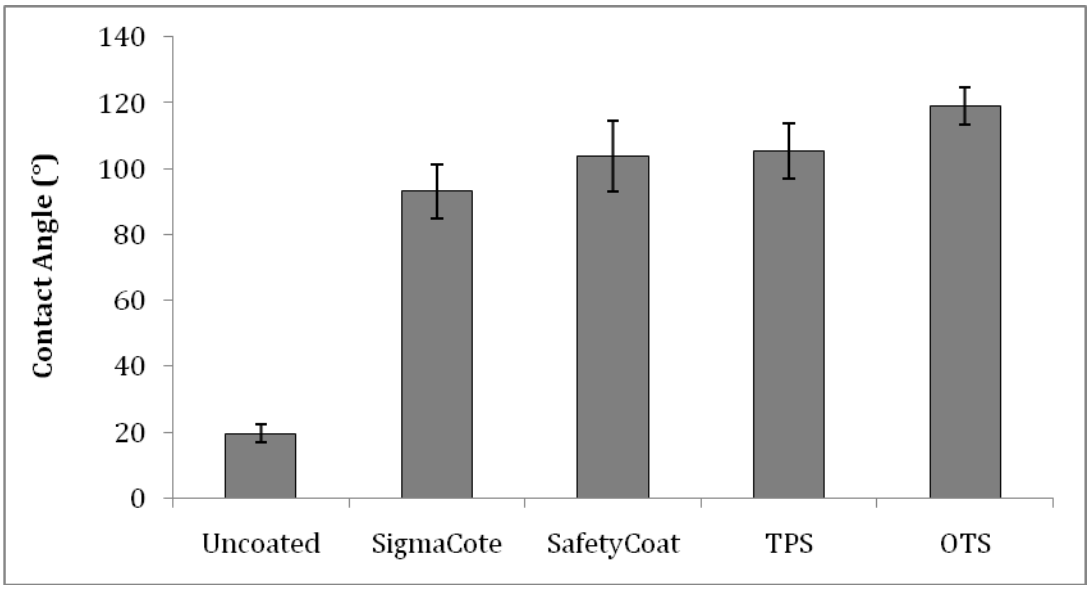


Figure 4.5: Graph showing the contact angles for water in the channel of microfluidic devices (Figure 4.1a) which had undergone various static passivation protocols. Uncoated microfluidic devices were used as a contact angle control. Error bars shown represent the standard deviation (n=4).

The greater the contact angle, the more hydrophobic the internal glass surface and so theoretically the less interaction there will be with the DNA polymerase and the more efficient the PCR reaction. Four silanisation techniques for treatment of the internal surfaces of the microfluidic device were investigated using SigmaCote™, Safetycoat™, OTS and TPS. Analysis of the efficiency of each of the techniques showed very little difference

in the contact angles obtained. As the contact angles were highest with OTS, this static passivation technique was initially chosen for preparation of microfluidic devices for PCR. It was soon found, however, that the silanisation treatment was subject to apparent degradation during the PCR thermal cycling process, as evident from an observed decrease in the contact angle (from ~115° to ~60°) following 40 cycles of DNA amplification. Such degradation of silanisation coatings has been reported by Felbel *et al.*, who showed that chlorotrimethylsilane, dichlorodimethylsilane, hexamethyldisilazane and trichloropropylsilane were all affected, albeit to different extents, by the DNA amplification process, resulting in cleavage of the silyl-ether bond.[118] Further investigation was conducted, therefore, in order to determine how each of the surface treatments were affected by the thermal cycling process. Following static passivation, the microfluidic devices were thermally cycled, using Peltier heating, and the contact angle recorded every 5 cycles (Figure 4.6).

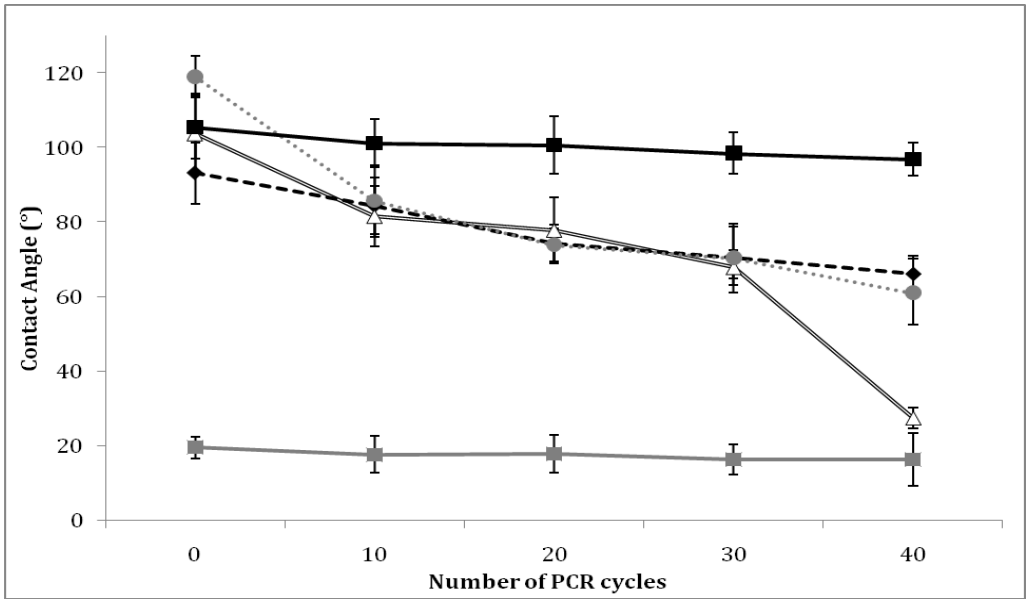


Figure 4.6: Graph showing the contact angles for water in the channel of microfluidic devices which have undergone thermal cycling. Static passivation methods applied were SigmaCote™ ( ), SafetyCoat™ ( ), OTS ( ) and TPS ( ). Uncoated microfluidic devices ( ) were used as a contact angle control. Error bars shown represent the standard deviation (n=4).

Analysis of the contact angles showed that SigmaCote™, SafetyCoat™ and OTS silanisation treatments were all susceptible to degradation during the thermal cycling process as observed by a decrease in the contact angles recorded. While TPS did not exhibit the highest contact angle initially, it was found to display the most consistent results during the thermal cycling process and so was chosen for passivation of microfluidic devices in all subsequent experiments. It has been hypothesised that enhanced stability of silanisation reagents, during DNA amplification can be achieved by formation of multi-layers by cross polymerisation of additional chlorine substituents.[118]

By comparing at the chemical structures of the different silanisation reagents it is hypothesised that TPS ($CF_3(CF_2)_5(CH_2)_2SiCl_3$) exhibits the greatest stability due to the presence of fluorine atoms, which will form more stable bonds due to higher electronegativity than chlorine atoms, and a flexible chain reducing steric hindrance. OTS ($C_{18}H_{37}Cl_3Si$) is the next most stable silanisation coating and contains in total three chlorine atoms. SigmaCote™ ($Cl[Si(CH_3)_2O]_3Si(CH_3)_2Cl$) and SafetyCoat™ ($(CH_3)_3SiCl$) do not have additional chlorine substituents and also have bulky chemical structures increasing steric hindrance and therefore reduces the number of molecules which can be attached to the microfluidic surface. In addition, the structure of TPS shares similar properties with Teflon® which has been demonstrated to be a contained adsorption material i.e. one which exhibits rapid saturation of DNA polymerase adsorption.[175] In order to provide the optimum conditions for performing DNA amplification on the microfluidic device a combination of static passivation, by TPS, and dynamic passivation, by a mixture of BSA, PVP and Tween-20 was chosen.

4.3.3 Analysis of Thermal Cycling Profiles

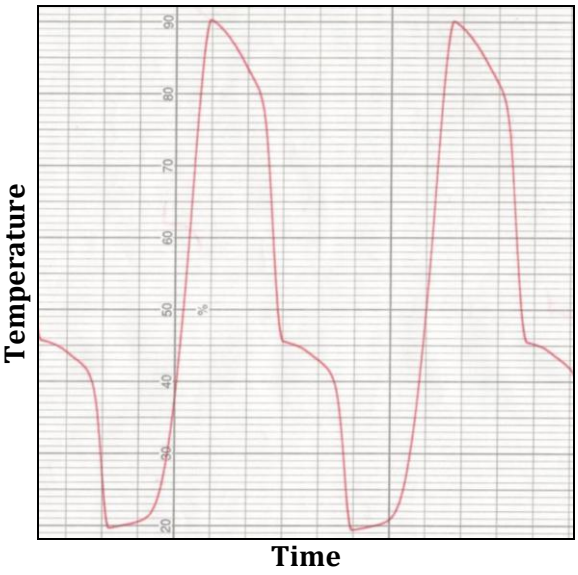
Once a suitable methodology had been developed to ensure that the microfluidic devices used were compatible with the DNA amplification process, a comparison of the different

heating systems was carried out. The thermal cycling profile of the heating system, including temperature ramping rates, is important in determining the efficiency of the DNA amplification process. For achieving efficient thermal cycling for successful DNA amplification it is important to reach a compromise between obtaining fast heating rates and preventing overshoot at the desired temperatures. In particular, if a temperature overshoot occurs at the denaturing temperature (94°C) it could lead to irreversible damage of the DNA polymerase or even boiling of the sample. In addition, undershooting of the temperature during cooling to the annealing temperature can result in non-specific binding of the primers leading to generation of spurious PCR products.

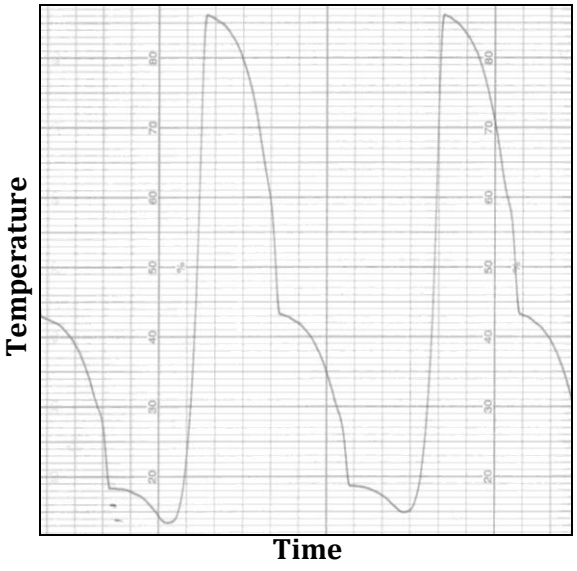
As such, the thermal cycling profiles for two different heating methods, Peltier and microwave, were evaluated and compared against the conventional PCR instrument. All three systems were set to cycle through the following temperatures; 94°C, 60°C and 72°C with 30 second hold times at each temperature. The thermal cycling profiles were monitored using an external fine gauge K-type thermocouple [Omega, UK] attached to a BD111 chart recorder [Kipp & Zonen, UK] (Figure 4.7).

As can be seen from the thermal cycling profiles in Figure 4.7, there is a significant difference between the contact (a-c) and non-contact (d) heating methodologies. The PCR instrument (a) shows no overshooting of temperatures but does not exhibit flat temperature holds, particularly at the DNA denaturation temperature. Both Peltier systems (b&c) have a slight undershoot at the annealing temperature, with the enclosed system (c) producing better results at the DNA denaturing temperature with a more defined hold. The profile of the microwave heating system (d) is drastically different, with faster heating and cooling rates and additionally there was no overshooting observed.

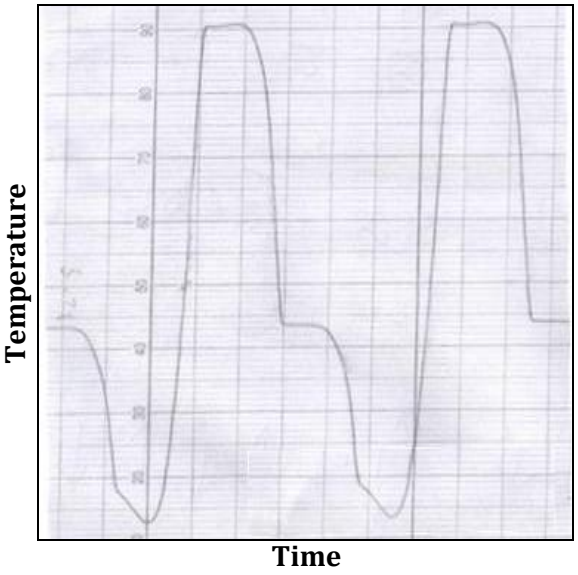
a) TC-312 PCR instrument



b) open bench-top Peltier heater



c) integrated genetic analyser Peltier heater



d) microwave heating system

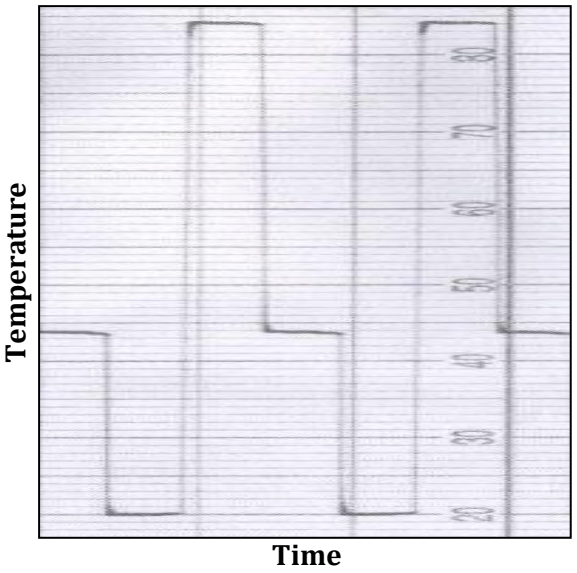


Figure 4.7: Thermal cycling profiles as determined using an external K-type thermocouple and chart recorder for a) TC-312 PCR instrument; b) open bench-top Peltier heater; c) integrated genetic analyser Peltier heater and d) microwave heating system.

As the PCR instrument does not exhibit any temperature plateau, it is possible that hold-times on the Peltier and microwave heating systems could be reduced to remove the plateau phases at each temperature, thus mimicking the thermal cycling profile of the PCR instrument. Such changes could significantly reduce the overall cycling time on the microfluidic system.

The thermal cycling information can be used to calculate heating and cooling ramping rates as well as giving an indication as to the precision of temperature holds (Table 4.4). The Peltier heating system used in both the bench-top and integrated genetic analyser exhibits very similar properties to the commercial thermal cycler, as both rely on block contact heating. Significantly increased heating and cooling rates were observed with the microwave heating system compared to the contact block heating methods. In addition, the control i.e. maintenance of a given temperature, was improved as can be seen from the temperature variation during holds.

Thermal cycler	Heating rate (°C/second)	Cooling rate (°C/second)	Precision hold (°C)
TC-312 PCR instrument	0.5	0.5	± 0.5
Open bench-top Peltier heater	0.5	1.5	± 0.1
Integrated genetic analyser Peltier heater	1	1	± 0.1
Microwave heating system	65	58	± 0.05

Table 4.4: Analysis of heating and cooling rates, as well as precision hold temperatures, for the different thermal cycling systems evaluated.

When using a microfluidic device for performing DNA amplification, as opposed to standard polypropylene tubes, there is an increase in thermal mass which needs to be considered. When microwaves were used, dielectric heating ensured that the entire microfluidic device was heated to a uniform temperature. By comparison, the size and

position of the Peltier heater creates a thermal gradient across the microfluidic device. The thermal diffusion time can be calculated using:

$$t = \frac{x^2}{\alpha} \qquad \textbf{(Equation 4.1)}$$

where t is the diffusion time (seconds), x is the thickness for the heat to diffuse (m) and α is the thermal diffusivity (m^2/second). The thermal diffusivity of a substrate is given by:

$$\alpha = \frac{k}{\rho c_\rho} \qquad \textbf{(Equation 4.2)}$$

where k is the thermal conductivity (W/(m·K)), ρ is the density (kg/m^3) and c_ρ is the specific heat capacity (J/kg·K). For the microfluidic system described here the time taken for the temperature of the DNA amplification chamber to equilibrate with the temperature of the Peltier heater is:

$$\alpha = \frac{1.1}{2200 \times 830} = 6.02 \text{ x } 10^{-7} \text{ m}^2\text{/second}$$

$$t = \frac{(0.001)^2}{6.02 \times 10^{-7}} = 1.66 \text{ seconds}$$

The time taken is significantly less than the hold times required for PCR thermal cycling at each temperature and so, even though there is a slight lag while the DNA amplification chamber reaches the desired temperature, equilibration occurs fast enough at the given parameters to support successful PCR.

4.3.4 DNA Amplification using Peltier Heating in a Microfluidic Device

Once the thermal cycling parameters of the Peltier heating system had been established, the suitability for performing PCR was investigated. DNA amplification in conventional

PCR instruments is usually carried out in either 0.2 ml or 0.5 ml polypropylene tubes. A comparison, performing PCR in these 0.2 ml polypropylene tubes, was carried out on both the conventional PCR instrument and Peltier heating system. DNA amplification, of the Amelogenin locus, was carried out according to the methodology given in Chapter 4.2.3. PCR samples were run in parallel on both systems for between 25 and 35 thermal cycles (Figure 4.8). A minimum of 28 thermal cycles were required to produce sufficient PCR products so they could be visualised using slab-gel electrophoresis. The PCR product bands observed from those samples amplified using the Peltier system were brighter than those amplified using the conventional PCR instrument, indicating that for these identical sample types, this method of thermal cycling is the more efficient. While hold time for the DNA denaturing temperature was set to 30 seconds for both the conventional PCR instrument and the Peltier heating system, the thermal cycling profiles show that the actual time at the desired temperature is longer on the Peltier heating system which demonstrated a more defined hold. Therefore it is possible that more complete DNA denaturation is occurring using the Peltier system resulting in a more efficient amplification reaction.

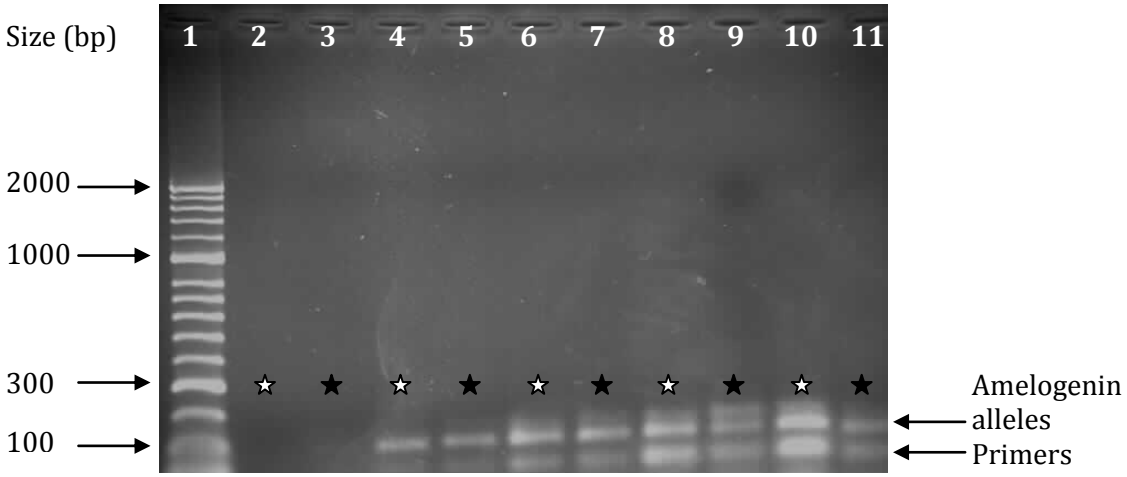


Figure 4.8: Comparison of DNA amplification on a Peltier heater (☆) and a conventional PCR instrument (★). UV transilluminator image of PCR products after varying cycle numbers, following slab-gel electrophoresis. Lane 1: DNA size ladder; 2 & 3: 25 cycles; 4 & 5: 28 cycles; 6 & 7: 30 cycles; 8 & 9: 32 cycles; 10 & 11: 35 cycles.

Following successful amplification of the Amelogenin locus in a 0.2 ml tube on the bench-top Peltier system, the same locus was amplified in the microfluidic device (Figure 4.1a) on the Peltier system contained within the integrated genetic analyser. PCR samples were again prepared according to the method given in Chapter 4.2.3. As shown in Figure 4.9, successful amplification of the Amelogenin locus on the microfluidic device was achieved.

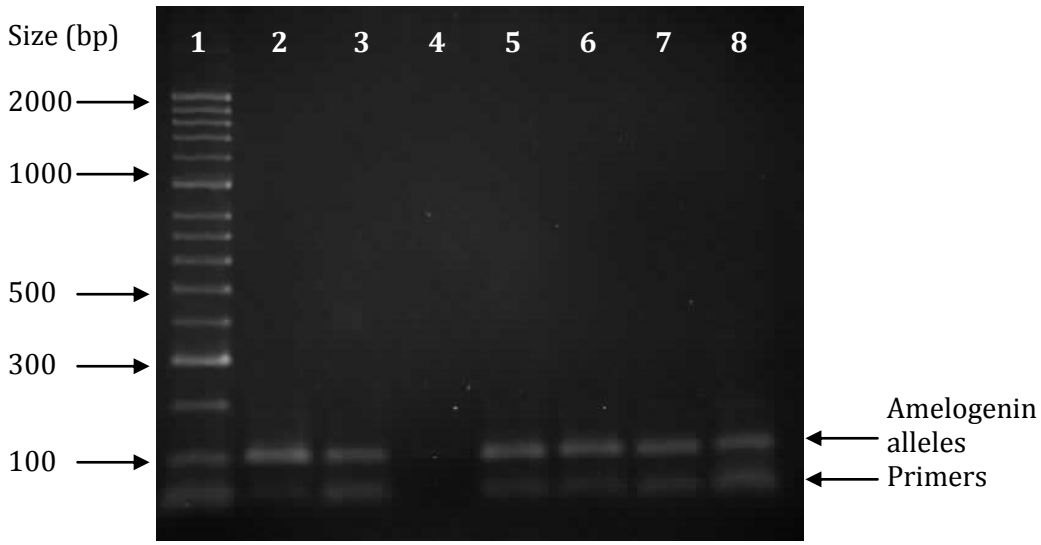


Figure 4.9: Single locus amplification on a microfluidic device. UV transilluminator image of PCR products amplified using a Peltier heating system contained within the integrated genetic analyser. Lane 1: DNA size ladder; 2: positive control performed on bench-top Peltier; 3: positive control performed on standard PCR instrument; 4: negative no DNA control; 5-7: 'push-through' samples performed on PCR instrument to check silanisation coating; 8: DNA amplified on the microfluidic device.

PCR amplification of all nine individual forensically relevant loci was also carried out on microfluidic devices in the integrated genetic analyser. Production of alleles ranging from approximately 100 bp to 400 bp was achieved. For each individual locus, the observed allele sizes, as confirmed using capillary electrophoresis, were recorded. The allele

designations were given accordingly based on the number of repeat sequences present, as determined by the length of the products (Table 4.5).

Locus	Expected Size Range (base pairs)	Observed Allele Sizes (base pairs)	Designation
Amelogenin	106-112	106	XX
D3 S1358	119-143	121, 135	15, 17
vWA	123-171	151, 155	17, 18
TH01	152-195	164, 168	6, 7
D8 S1179	203-251	227, 231	13, 14
D21 S11	203-273	223, 227	29, 30
D16 S539	264-304	288, 292	11, 12
D18 S51	290-366	306, 322	12, 16
FGA	318-460	344, 362	21.2, 26

Table 4.5: Expected allele size range and observed allele size, including designation, of samples amplified on microfluidic device using Peltier heating.

Once PCR amplification of the individual loci on the microfluidic device had been achieved, the next stage was to perform multiplex analysis using the commercially available SGM Plus™ kit which contains primers for 10 STR loci plus the Amelogenin sex marker. Amplification of the SGM Plus™ kits was performed on the microfluidic device using PCR reagents and temperatures according to the manufacturer's recommendations (Chapter 4.2.3). However, following analysis by capillary gel electrophoresis, a complete DNA profile was not produced. A range of experimental variables were evaluated to try and achieve amplification of the SGMplus™ loci on the microfluidic system presented here including: different denaturing temperatures, different annealing temperatures, adjusting the hold times, addition of further dynamic passivation agents such as BSA and replacing

the Hot-Start step with an increased number of cycles. The series of experiments performed still failed to produce a complete DNA profile, with a maximum number of 7 amplified loci obtained simultaneously. Preferential amplification of the lower molecular weight loci was observed (Figure 4.10).

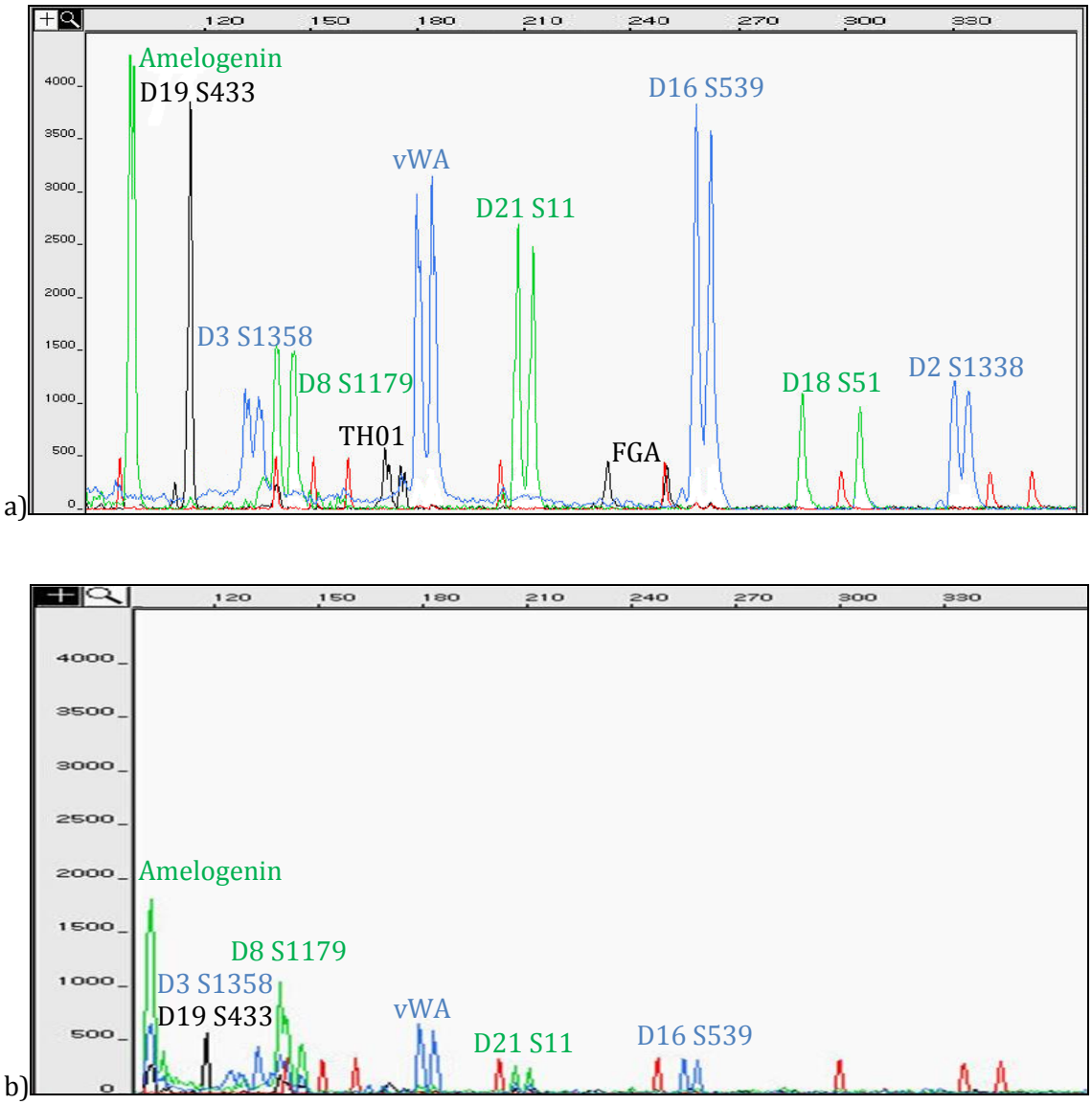


Figure 4.10: a) Electropherogram showing complete SGM Plus™ DNA profile, amplified on a conventional PCR instrument. Size (base pairs) is shown on the x axis and fluorescent intensity (arbitrary units) is shown on the y axis. Red peaks represent the ROX labelled DNA size standard; b) Electropherogram showing partial amplification of SGM Plus™ loci on the microfluidic device, with additional BSA and an increased DNA extension time of 90 seconds.

Preferential amplification of lower molecular weight loci potentially suggests either a degraded DNA sample or insufficient DNA extension time. Positive control samples, ran on a conventional PCR instrument, produced full SGMplus™ DNA profiles eliminating degraded DNA as the reason for the poor results on the microfluidic device. Increased DNA extension times (of up to 2 minutes) were also evaluated for PCR on the microfluidic device and showed no amplification of the larger molecular weight loci. Therefore further explanation was sought.

The SGM Plus™ kit consists of an AmpF*l*STR® PCR Reaction Mix, an AmpF*l*STR® SGM Plus™ Primer Set and an AmpliTaq Gold® DNA polymerase which are added together in the presence of DNA, the solutions of which are under proprietary constraints and so the exact compositions are unknown. This means it is difficult to alter individual reagent concentrations, such as trying to increase the primer concentrations of those loci which have not amplified. As a preliminary investigation an individual set of primers, for amplification of the TH01 locus, was added to the SGMplus™ kit and amplification performed on the microfluidic device. While the SGMplus™ loci failed to amplify, a strong reaction product for the TH01 locus was observed. This confirms that the AmpF*l*STR® PCR Reaction Mix and AmpliTaq Gold® DNA polymerase are both compatible with DNA amplification in microfluidic systems and points to primer concentration as the source of the problem.

For further investigation of multiplex PCR within a microfluidic device, an in-house multiplex was produced in which the concentration of individual primers sets could be altered to try and elucidate the issues involved with performing multiplex PCR analysis on a microfluidic device. As SGM Plus™ kit primer sequences are under proprietary constraints, primer sequences for those loci which are found in parallel in the Powerplex® 16 System [Promega, US], used by forensic services in the United States, were used as these sequences are readily available in the public domain. While nine out of the eleven SGM Plus™ kit loci are also present in the Powerplex® 16 System and so the primer

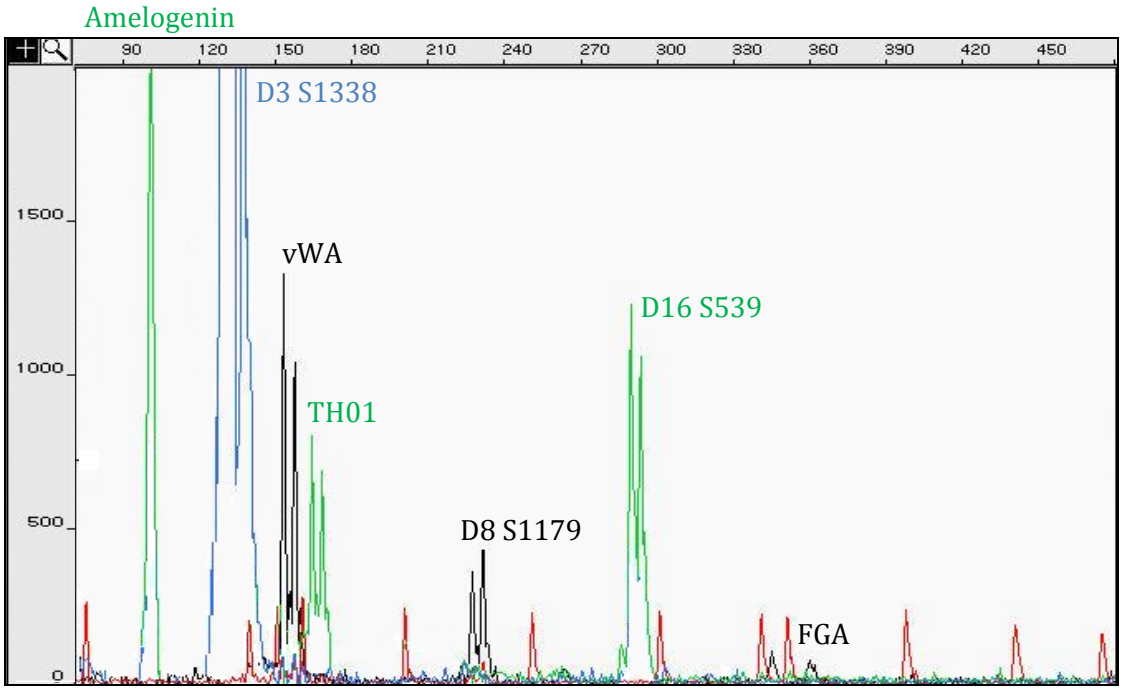
sequences are available, two of them are not (D2 S1338 and D19 S433) and so these were not included in the multiplex. All further work, relating to multiplexing, was carried out using eight STR loci and the Amelogenin sex marker (see Table 2.3 for details).

Before attempting to perform multiplex PCR on the microfluidic device it was necessary to produce a working 9-plex, using the selected primer sets, that was optimised and robust. Following optimisation of the conditions for each individual primer set (see Chapter 4.3.1) a multiplex was gradually built up. As the primer sequences were obtained from the PowerPlex16® System, certain information pertaining to the optimum conditions for the multiplex reaction were already known, for example, the use of a 60°C annealing temperature. However, due to proprietary constraints details of the biochemistry are more limited such as $MgCl_2$ and relative primer concentrations. The optimised protocol required in order to produce a balanced peak height DNA profile with no non-specific products was determined experimentally, using a conventional PCR instrument, and found to be 1 x GoTaq® buffer, 1 mM $MgCl_2$, 200 µM each dNTPs, 0.2 µg/µl BSA, 0.01% (w/v) PVP, 0.1% (v/v) Tween-20 and 0.1 U/µl GoTaq® DNA polymerase (see Table 4.6 for primer concentrations). When the 9-plex was transferred onto the microfluidic device, allele drop-out of some of the larger molecular weight loci occurred (Figure 4.11). It was found that it was necessary to alter the relative primer concentrations for these larger molecular weight loci to enable a balanced 9-plex DNA profile to be produced on the microfluidic device (Table 4.6).

Locus	Expected Size Range (base pairs)	Concentration – conventional (µM)	Concentration – microfluidic (µM)
Amelogenin	106-112	0.2	0.1
D3 S1358	119-143	0.2	0.1
vWA	123-171	0.3	0.1
TH01	152-195	0.2	0.2
D21 S11	203-273	0.2	0.3
D8 S1179	203-251	0.3	0.4
D16 S539	264-304	0.3	0.2
D18 S51	290-366	0.3	0.4
FGA	318-460	0.5	0.6

Table 4.6: Comparison of primer concentrations used for performing multiplex PCR analysis in both conventional and microfluidic systems.

a)



b)

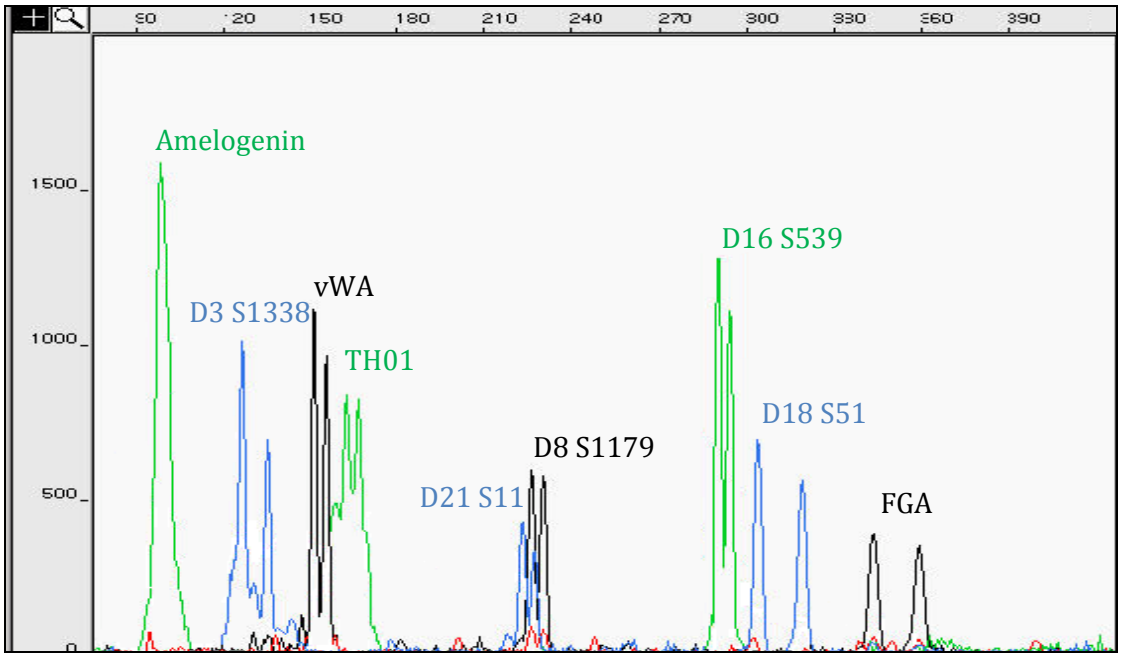


Figure 4.11: Electropherograms showing the DNA profile produced from amplification of the nine forensically relevant loci performed on a microfluidic device using a) standard PCR instrument primer concentrations and b) modified primer concentrations optimised for working on the microfluidic device. Size (base pairs) is shown on the x axis and fluorescent intensity (arbitrary units) is shown on the y axis.

When performing multiplex PCR analysis within the microfluidic device it was, in general, necessary to increase the concentration of primers for the higher molecular weight loci relative to the lower molecular weight loci. This result may go some way to explaining why it was not possible to directly use the SGMplus™ kit on the microfluidic system. By having the option to alter the primer concentrations a complete DNA profile has been produced. Multiplex PCR in microfluidic systems has not been widely reported in the literature, although recently published forensic STR analysis has also made use of a custom-made primer set rather than a commercially available DNA profiling kit.[193] The reasons for this are not stated, but the same group previously reported a device for CE separation of all 16 PowerPlex16® System loci,[209] so there are questions as to why the same kit was not used for combined PCR and CE, possibly due to the reasons reported here.

4.3.5 DNA Amplification using Microwave Heating in a Microfluidic Device

While the Peltier heating system described above enabled multiplex PCR analysis to be performed on a microfluidic device within the integrated genetic analyser, the heating and cooling rates do not offer any significant advantage over conventional PCR instruments. Both use contact heating blocks which, despite suffering from slow thermal transitions, are capable of high precision temperature control. Microwave heating systems have previously been used to perform DNA amplification of large sample volumes, up to 15 ml, for high throughput studies but their use in microfluidic systems has yet to be evaluated.[174]

The microwave heating system, described in Chapter 2.2.4, relies on radiofrequency (RF) dielectric heating of the entire microfluidic device rather than direct microwave heating of the PCR sample. This mechanism of heating results in greater temperature uniformity and therefore faster and more accurate temperature stabilisation.

Microfluidic devices (Figure 4.1b) were prepared with DNA amplification chambers of varying volumes to evaluate the capabilities of the microwave heating system for performing low volume PCR. A PCR reagent mixture was produced, as described in Chapter 4.2.3, for amplification of the Amelogenin locus. Once the microfluidic device was prepared it was placed into the microwave cavity. Tuning of the microwave cavity, with the microfluidic device in position, was achieved by adjusting the proximity of the two halves of the microwave cavity. The resonant frequency was monitored on an 8719D Network Analyzer [Hewlett Packard, UK] and was found to range from 7.85 to 8.11 GHz with an average of 8.02 GHz (± 0.06 GHz). A range of resonant frequencies are produced due to slight variations in the individual microfluidic devices used as a result of the manufacturing process.

Temperature feedback was provided using a K-type thermocouple attached to an oscilloscope which allowed heating and cooling rates to be monitored (Figure 4.6). Transition between temperatures was very tightly controlled using the microwave heating system resulting in no overshooting at any of the desired temperatures. In addition the control system allows real-time adjustment of the temperatures. The ability to so precisely reach the desired temperatures, in particular the primer annealing temperature, were found to have an impact on the DNA amplification reaction.

Prior to thermal cycling, a two minute initial incubation at 95°C was used to activate the GoTaq® Hot-Start DNA polymerase. A total of 28 PCR cycles were then carried out using 30 second hold times at 94°C, 60°C and 72°C. Successful amplification of the Amelogenin locus, from both male (XY) and female (XX) donors was possible, in the microfluidic device, in volumes as low as 1.1 µl (Figure 4.12).

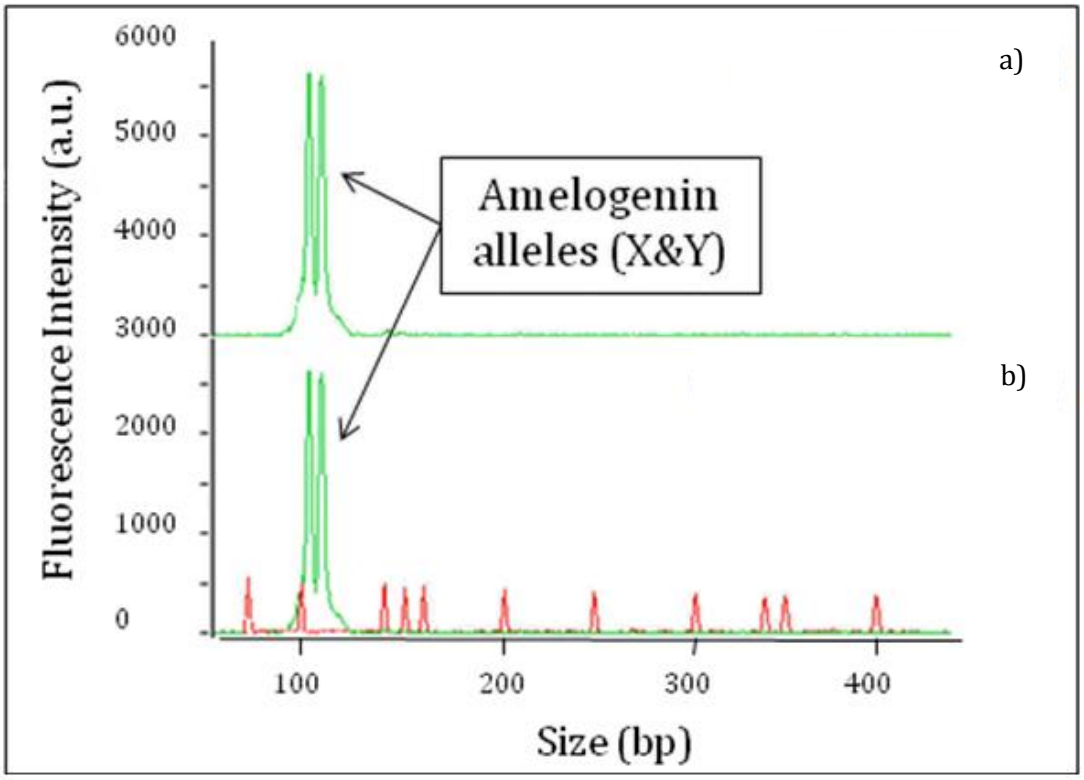


Figure 4.12: Electropherograms showing amplification of the Amelogenin locus, from a male donor, using a) a microfluidic device with the microwave heating system and b) a 0.2 ml polypropylene tube with the conventional PCR instrument.

An estimation of the DNA amplification efficiency of the microwave heating system, compared to a conventional PCR instrument, was carried out by examining the relative fluorescence intensity of the PCR products. The intensity of PCR products amplified on the microwave heating system was 100.3 ± 0.5% (n=4) compared to those produced on the conventional PCR instrument.

Due to time constraints optimisation of the microwave heating system was not possible during this programme of work. However, the fact that the efficiency was comparable with the conventional PCR instrument, with run times significantly reduced make this a viable option for the development of a portable microfluidic device capable of performing DNA profiling. The average power consumption of the microwave heating cavity during a

typical amplification process was around 500 mW. In the present system, cooling was provided using compressed air, controlled by a solenoid, providing cooling rates of 58°C/second. The inclusion of compressed air within a portable genetic analysis system may not be practical, however, it was shown that even just using convection, cooling rates of 31°C/second were possible. To reach the annealing temperature, from the denaturing temperature, takes 0.6 seconds with compressed air cooling and 1.1 seconds without. Over 28 cycles this results in just a 14 second time difference, therefore simply using convection in the portable genetic analysis system would not have a dramatic impact on the overall time for DNA amplification.

4.3.6 Real-time Monitoring of DNA Amplification

Real-time monitoring of the DNA amplification reaction provides information as to the initial starting concentration of DNA which has been added to the system. In an integrated genetic analysis system, this could prove a valuable asset. As the amount of DNA entering the device will vary depending upon the sample type, it would allow flexible control of the number of PCR cycles until a threshold amount of DNA is reached that is sufficient for the detection system to analyse.

As an example for preliminary studies, real-time monitoring of the amplification of the Amelogenin locus was monitored using the system described in Figure 2.5.3. By including a fluorescent intercalating dye, PicoGreen®, in with the PCR reagents, the fluorescence intensity, proportional to the amount of double-stranded DNA present, was monitored. A typical sigmoidal curve was produced which clearly shows the exponential phase, beginning around 20 cycles (Figure 4.13).

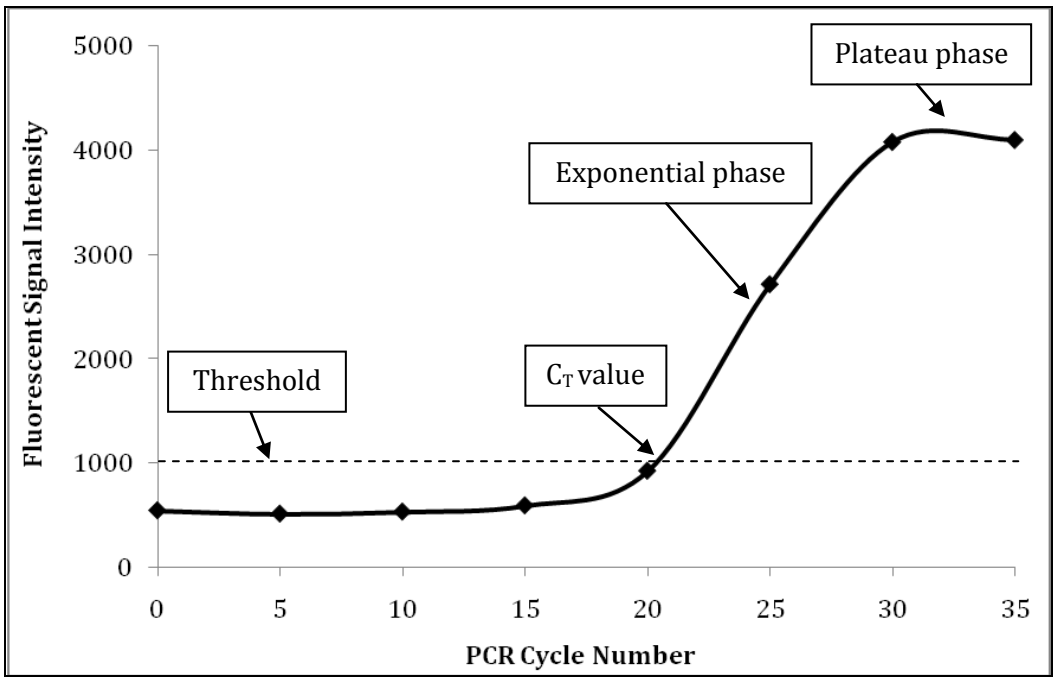


Figure 4.13: Real-time monitoring of DNA amplification of the Amelogenin locus on the microfluidic device, with monitoring of fluorescence intensity every cycle.

At 30 cycles a plateau effect is observed, typical of PCR, where DNA amplification is no longer exponential; this occurs as a result of a number of contributing factors including: reagent depletion, DNA polymerase degradation, inhibition by end products e.g. dsDNA and competition for reagents by non-specific products.[165] Monitoring of the reaction in such a way on the microfluidic device could be used as a control making sure the appropriate amount of amplification has occurred, depending on the initial amount of template DNA loaded, for adequate detection after electrophoretic separation.

As part of a supervised BSc student project where preliminary work was carried out by Elizabeth Hughes, real-time monitoring of PCR amplification using TaqMan™ style probes was also investigated. While the aim of the BSc project was not forensic STR analysis, it revolved around the principles of nucleic acid extraction and amplification on a microfluidic device. Briefly, the project involved looking at the levels of CYP1A2 mRNA in primary rat hepatocytes. CYP1A2 is a cytochrome P450 enzyme, which is upregulated in

the presence of toxic compounds facilitating their excretion from the organism concerned.[210] Monitoring of CYP1A2 mRNA levels in respect to the mRNA levels of a house-keeping enzyme, in this case glyceraldehyde-3-phosphate dehydrogenase (GAPDH), allows this upregulation to be examined giving an indication as to the toxicity of the compound.[210]

To confirm that 3-methylcholanthrene increases CYP1A2 production, RT-PCR was carried out on induced and non-induced primary rat hepatocytes as described in Chapter 2.5.3. End-point PCR analysis using capillary gel electrophoresis showed that, as expected, while GAPDH levels remained constant, CYP1A2 levels were increased in the induced primary rat hepatocyte population (Figure 4.14).

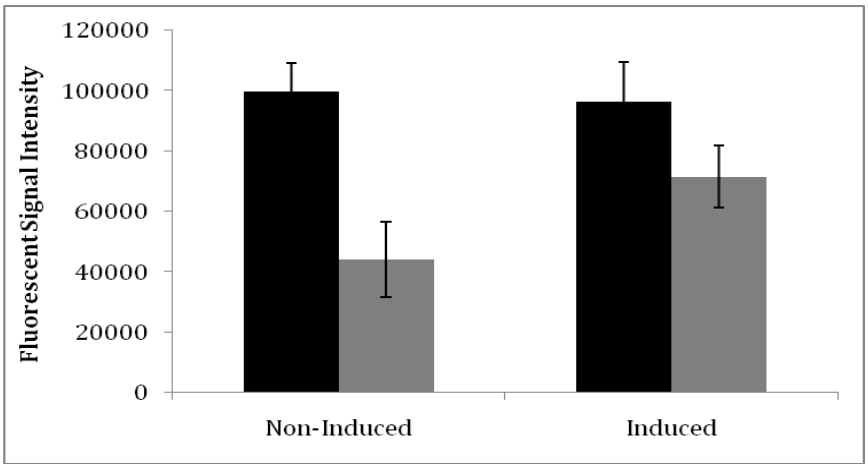


Figure 4.14: Comparison of induced and non-induced primary rat hepatocytes following RT-PCR in a conventional PCR instrument. Monitoring of the relative peak heights, using capillary gel electrophoresis, as an end-point measurement was used where GAPDH (■) was used as an internal control against CYP1A2 (▩) (n=3).

Real-time monitoring of RT-PCR on the microfluidic device was then performed using the same methodology as described for monitoring of the Amelogenin locus described above. In this case TaqMan™ style probes (Chapter 2.5.3) were used to allow the simultaneous monitoring of GAPDH and CYP1A2 amplification (Figure 4.15). As is consistent with

end-point PCR analysis, the fluorescence intensity of GAPDH reaches an arbitrary threshold (C_T) approximately 2 cycles before that of the CYP1A2, due to a higher initial starting concentration of mRNA molecules in the sample.

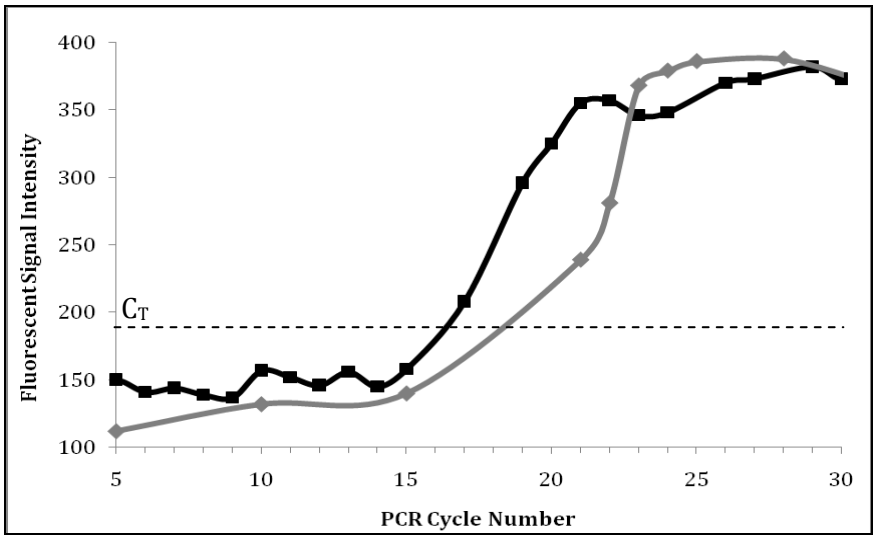


Figure 4.15: Graph showing the real-time monitoring of RT-PCR carried out on the microfluidic device using the 3-methylcholanthrene induced cell line. Both GAPDH (■) and CYP1A2 (■) levels were monitored using TaqMan™ style probes.

While the application described here is not related to forensic STR analysis, the technology could be applied to the integrated genetic analysis system. DNA quantification is used in conventional forensic genetic analysis laboratory protocols following DNA extraction so that the optimum amount of DNA is used for the amplification process; too much DNA can result in off-scale peaks on the electropherograms and too little can lead to incomplete DNA profiles. Currently in the microfluidic device presented here, the amount of DNA transferred to the PCR chamber is determined by the capacity of the monolith and the DNA extraction efficiency. The use of real-time monitoring of PCR provides a viable alternative for ensuring an optimum amount of DNA amplification has occurred enabling a complete DNA profile to be obtained following capillary gel electrophoresis on the microfluidic device.

4.4 Summary

A range of different heating methods have been reported in the literature for performing thermal cycling on microfluidic devices for the purpose of DNA amplification (see Table 1.2). Reported heating and cooling rates vary dramatically depending on the type of heating method employed (Table 4.7).

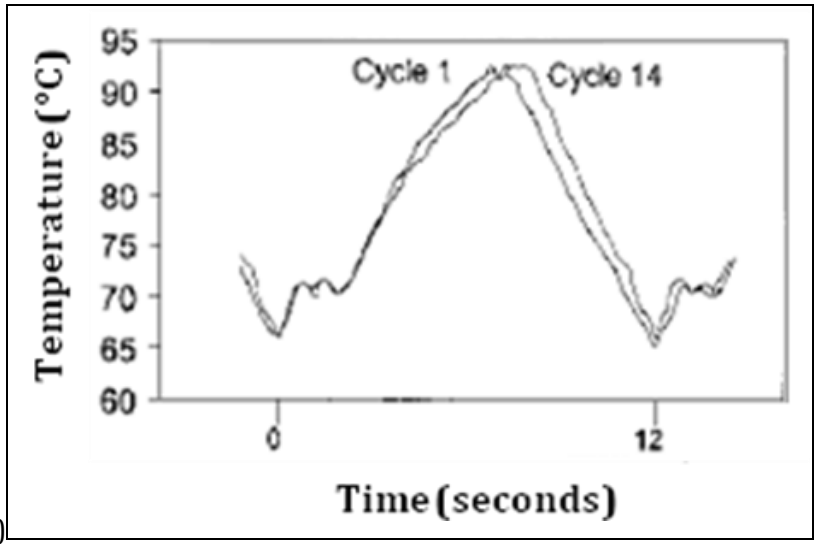
	Heating Rate (°C/sec)			Cooling Rate (°C/sec)		
Heating Method	Min.	Max.	Average	Min.	Max.	Average
Block heater [102, 104, 136]	2	16	8	1	16	7
Induction heating [211, 212]	7	16	11	4	10	7
IR heating [97, 105, 108, 213]	10	65	28	6	20	14
Thin film resistive heater [17, 115-117, 121, 122, 124, 131]	5	80	35	4	74	27
Microwave heating	-	-	65	-	-	58
Thin film PCB heater [101]	-	-	175	-	-	125

Table 4.7: Comparison of heating and cooling rates of different thermal cycling method employed for DNA amplification on microfluidic devices. The highlighted rows represent those thermal cycling techniques which have been evaluated as part of this chapter.

The Peltier heating systems used here provided heating and cooling rates very similar to conventional PCR instruments (~2°C/second) but were easily amenable to performing single and multiplex DNA amplification in a microfluidic system. Evaluation of the microwave heating system demonstrated that faster heating and cooling rates of 65°C/second and 58°C/second respectively were achieved. While the microwave heating system described here is not the fastest thermal cycling method reported in the literature

for DNA amplification in microfluidic systems, transitions between the temperatures required for PCR could be achieved in less than a second with a high degree of accuracy when held at temperature (±0.05°C). The thermal cycling profile of the microwave heating system was shown to be highly reproducible in comparison to that previously reported for IR heating (Figure 4.16).

A)



B)

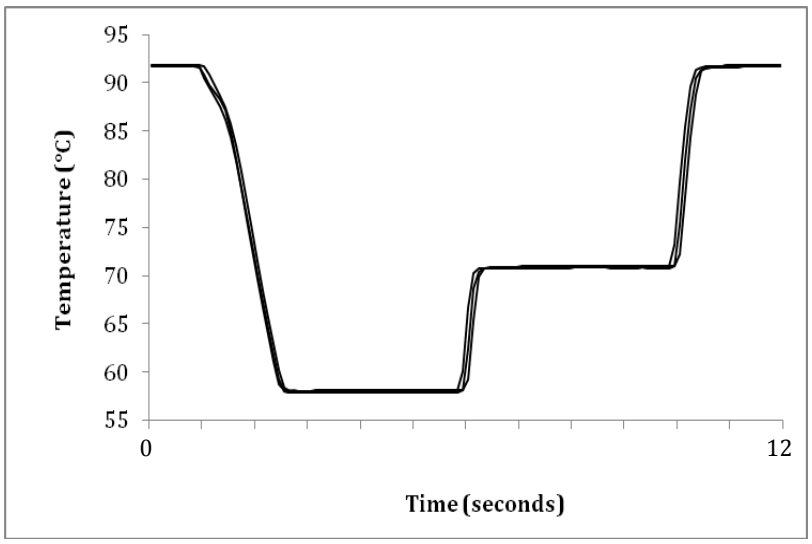


Figure 4.16: Comparison of thermal cycling reproducibility. A) Overlaid thermal profiles for cycles 1 and 14 using IR heating.[177] B) Overlaid thermal profiles for cycles 10, 20 and 30 for the microwave heating system presented here.

Currently, using the microwave system presented here, 28 cycles can be performed in 42 minutes, which although representing a significant time saving compared to conventional PCR instruments (up to 150 minutes), is not one of the shortest analysis times reported in the literature. While the thermal transition is rapid, in these examples the hold times used at each temperature are kept constant at 30 seconds to enable direct comparison between the different heating methods. Such hold times are usually required to allow sufficient time for the temperature in the PCR sample to equilibriate.[214] Due to smaller fluidic volumes in microfluidic systems, thermal cycling is no longer restricted by the transition between temperatures but by the reaction kinetics which form the rate limiting step. DNA polymerase enzymes have a processivity of 60-100 nucleotides/sec at 72°C [142] so using the set of loci selected for analysis here, with alleles theoretically ranging from 106 bp to 460 bp in size, DNA extension could theoretically occur within ~5 seconds. Using hold times of 5 seconds for each of the cycling temperatures, 28 PCR cycles could potentially be completed in 7 minutes using the microwave heating system.

A range of DNA amplification chambers with different volumes were evaluated. Due to the high surface area to volume ratio, surface passivation was required to prevent DNA polymerase adsorption. A combination of silanisation, using TPS, and dynamic passivation, using 0.2 μg/μl BSA, 0.01% (w/v) PVP and 0.1% (v/v) Tween-20, was shown to be optimum in preventing DNA polymerase adsorption. The minimum sample volume successfully amplified was 1.1 μl, using both the microwave and Peltier heating systems. While microfluidic PCR has been demonstrated in volumes of as little at 86 pl there are issues with the so called 'world-to-chip interface' which have limited the volume used here. The microfluidic device must be able to accept sufficient sample volume to obtain at least 1 ng of DNA for PCR following DNA extraction e.g. from a buccal swab. Also, prior to development of the integrated separation and detection system (as part of the overall project), conventional analysis of PCR products using slab- or capillary gel electrophoresis was required therefore there is a minimum sample volume required for detection in such systems. Although, later on real-time monitoring of PCR DNA amplification was shown to

be possible on the microfluidic device using either an intercalating dye or TaqMan™ style probes. Even so the use of such relatively 'large' microfluidic PCR volumes offers considerable advantage over conventional systems. One area of consideration is cost, for example, simply looking at the cost of the DNA polymerase enzyme for performing a 1.1 µl reaction, £0.03, compared to a standard 50 µl reaction, £1.34, shows considerable financial savings.

Microwave heating has previously been reported for DNA amplification in reaction vessels of 15 ml. While successful amplification of a 53 bp fragment was achieved within 127 minutes there were limitations to the system. The microwave cavity was not capable of cooling or internal temperature measurement. Therefore, cooling required the PCR tube to be manually transferred to a heat block of lower temperature for the annealing phase and to achieve heating the irradiation pulse had to be determined empirically.[215] The microwave heating system reported here demonstrates DNA amplification on a microfluidic device with integrated cooling and temperature measurement. In addition the system was shown to produce successful amplification of 106 and 112 bp alleles in 42 minutes, representing a major advance in the development of microwave systems applicable for PCR within a microfluidic environment.

Direct use of the SGMplus™ kit on the microfluidic DNA amplification system failed to generate a full DNA profile due to inadequate primer concentrations. However, production of a complete DNA profile from an in-house 9-plex was achieved. The degree of discrimination provided by the SGMplus™ kit and the 9-plex PCR kit developed here are 2.6×10^{-18} and 2.2×10^{-14} respectively (Table 4.8). While a greater degree is obviously provided by the SGMplus™ kit, due to the increased number of loci examined, both provide a degree of discrimination greater than 1 in a billion which is required for legal purposes. Therefore the DNA amplification system described here fulfils the aim of producing a microfluidic device capable of producing a forensic DNA profile.

Locus	Observed Alleles - SGMplus™ (bp)	Observed Alleles - 9plex (bp)	Allele Designation	Frequency
Amelogenin	106	106	XX	0.5
D2 S1338	329	n/a	24	0.142
	333	n/a	25	0.111
D3 S1358	125	121	15	0.265
	133	135	17	0.195
D8 S1179	147	227	13	0.343
	151	231	14	0.199
D16 S539	257	288	11	0.289
	261	292	12	0.288
D18 S51	284	306	12	0.158
	300	322	16	0.122
D19 S433	118	n/a	12	0.087
	118	n/a	12	0.087
D21 S11	206	223	29	0.205
	210	227	30	0.271
FGA	232	344	21.2	0.002
	250	362	26	0.029
TH01	172	164	6	0.243
	176	168	7	0.178
vWA	180	151	17	0.268
	184	155	18	0.209
Degree of discrimination for SGMplus™				**2.6×10^{-18}**
Degree of discrimination of 9plex				**2.2×10^{-14}**

Table 4.8: Observed alleles for the SGMplus™ and custom-made 9-plex. The frequency of each allele in the UK Caucasian population is given, showing overall degree of discrimination provided by each system.

5 Integrated DNA Extraction and Amplification

5.1 Introduction

The previous two chapters have focused on optimisation of two of the individual processes required for DNA profiling, namely DNA extraction and amplification. One of the benefits of microfluidic technology is that is allows integration of multiple processes on a single device. The aim of the work described in this chapter is to develop an integrated methodology for combining DNA extraction and amplification in a single device, which is compatible with a purpose built control platform. While the integration of PCR with real-time detection or capillary electrophoresis for analysis of PCR products has received substantial attention in the literature, much less has been reported on combining PCR with upstream processes.[216] Although there have been a few reports of DNA extraction incorporated prior to PCR,[81, 190-192] upstream processes can also refer to the isolation of specific cell types and/or cell lysis without subsequent DNA extraction.[28, 187, 188]

In order to successfully combine DNA extraction and amplification on a single microfluidic device there are a number of important issues which need to be taken into account.[72] Firstly, the solid-phase matrix must be confined to the DNA extraction chamber, particularly as the high surface area to volume ratio created by the matrix can have a detrimental effect on PCR due to adsorption of the DNA polymerase. As demonstrated in Chapter 3, confinement of the thermally activated potassium silicate monolith in the DNA extraction chamber can be achieved through chamber geometry and the use of glycerol. Secondly, contamination of the PCR chamber with potential inhibitors from the DNA extraction process e.g. guanidine hydrochloride and isopropanol, must be prevented. Finally, any surface coating used to prevent DNA polymerase adsorption in the PCR chamber must be isolated from the DNA extraction region. These latter two issues are ones which have not previously been addressed through optimisation of individual reactions and so will be dealt with as part of this chapter.

Initial evaluation of a hydrodynamically-driven system for the integration of DNA extraction and amplification showed inherent problems with such methodology, particularly relating to contamination issues. Therefore the focus of the work presented here is an evaluation of an electrokinetically-driven system. Progression from a hydrodynamic to an electrokinetically-driven flow system does not significantly change any of the operational parameters identified but does require each of the processes to undergo re-optimisation. In addition, the development of a portable integrated genetic analyser would benefit from storage of all the necessary reagents on the microfluidic device, as this minimises the number of steps required for operation at the point-of-use. The storage of reagents, for performing DNA extraction and amplification within the microfluidic device, by encapsulation in agarose was investigated.

5.2 Experimental

5.2.1 Microfluidic Device Design for Integrated Genetic Analysis

Microfluidic devices were manufactured by Dr. Steve Clark at the University of Hull, according to the protocol given in Chapter 2.1. DNA extraction and amplification could both be performed on the microfluidic device shown in Figure 5.1. In addition this integrated device was also designed for subsequent capillary gel electrophoresis analysis of PCR products.

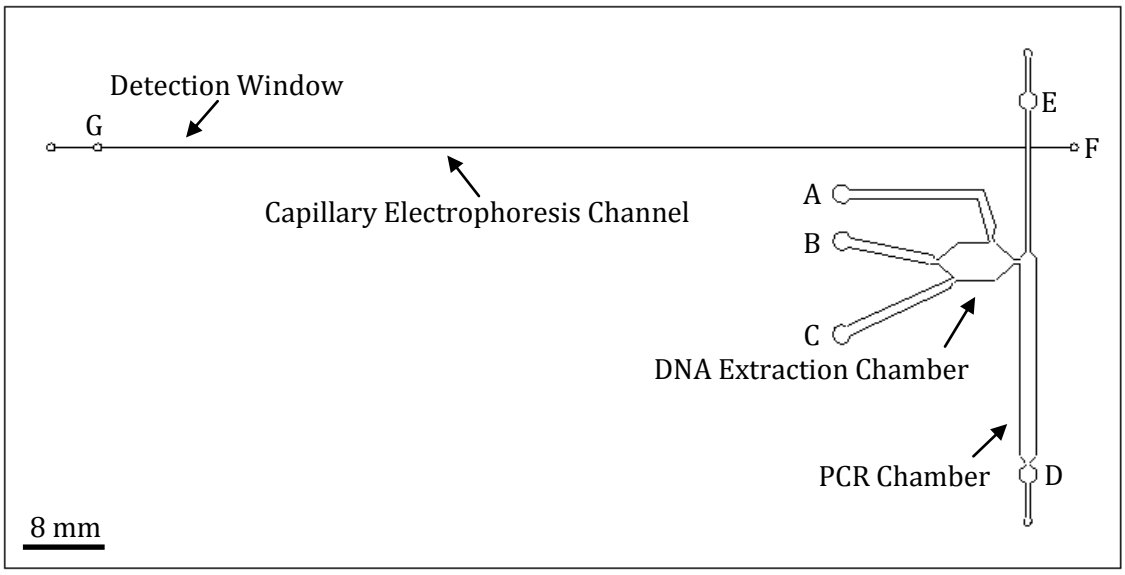


Figure 5.1: Schematic showing the design of the full microfluidic device for DNA extraction, DNA amplification, electrophoretic separation and fluorescence detection. All features are etched into the top plate to a depth of 100 µm. The 1 mm ports, shown by A-G, allow carbon electrodes to be attached to the microfluidic device. (N.B. While the design of the full device has been included for completeness, it is only the DNA extraction and DNA amplification sections which are examined here).

5.2.2 DNA Extraction & Amplification using Electrokinetic Pumping

Integrated DNA extraction and amplification was carried out using electro-osmotic pumping (EOP) for movement of reagents through the microfluidic device. The thermally activated silica monolith in the DNA extraction chamber has dual functionality, acting as both the solid-phase for DNA adsorption and as a pump for electro-osmotic flow (EOF). The microfluidic device was pre-loaded with all the necessary reagents for performing both procedures. Storage of reagents for DNA extraction and amplification within the device was facilitated by encapsulation in a gel format to provide increased structural integrity.

A molecular biology grade low melting temperature (LMT) agarose [Sigma-Aldrich, UK] was used in all experiments as it ensures the reagents will be in a molten format during each phase of the thermal cycling reaction, including the lower primer annealing temperature. Standard agarose gels, as used for slab-gel electrophoresis, have a fusion point of ~90°C and a gelling temperature of ~40°C whereas LMT agarose gels have a fusion point of ~65°C and a gelling temperature of ~25°C.

For the DNA extraction wash step, 1.5% (w/v) LMT agarose was dissolved in either isopropanol or ethanol by heating to 60°C. A range of percentage (v/v) alcohol gels were investigated as described in Chapter 5.3.2. The wash gel was then manually injected into channel A on the microfluidic device. For the DNA extraction elution step, 1.5% (w/v) LMT agarose was dissolved in either water or TE buffer by heating to 60°C. A range of TE buffer concentrations were investigated as described in Chapter 5.3.2. The elution gel was then manually injected into channel B on the microfluidic device (Figure 5.2).

For DNA amplification, LMT agarose was dissolved in purified water, at a range of % (w/v) for evaluation by heating to 60°C. Once dissolved, the agarose gel was allowed to set, by storing at 4°C, prior to addition of the PCR reagents shown in Table 2.1. Following addition of the PCR reagents, the agarose gel was melted again to facilitate encapsulation

of the PCR reagents and was then manually injected onto the microfluidic device in the DNA amplification chamber (Figure 5.2). As part of a stability study (Chapter 5.3.3) the gel encapsulated PCR reagents were stored at room temperature, 4 °C or -20 °C.

Once all the necessary reagents had been loaded onto the microfluidic device, the inlets were then sealed using injection molded carbon containing polystyrene electrodes and the microfluidic device stored at 4°C until required.

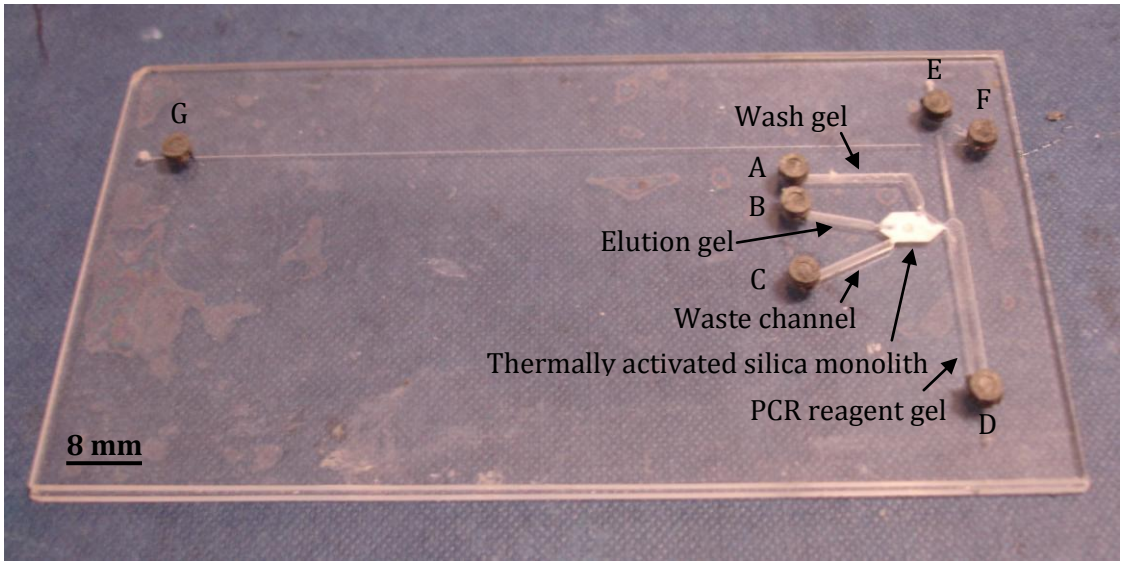


Figure 5.2: Photograph showing set up of microfluidic device for EOP using carbon electrodes, including the silica monolith in the DNA extraction chamber.

The genomic DNA sample (either pre-extracted using a QIAamp® DNA Micro Kit or directly from a buccal swab) in GuHCl solution was transferred manually onto the monolith using a displacement pipette and relied on capillary action to draw the DNA loading solution into the empty waste channel (C). Electrokinetic movement was controlled using the integrated genetic analyser in which copper pogo-pins make contact with the electrodes on chip. The pogo-pins are attached to 1 kV power supplies [EMCO, US] which can be programmed to provide voltages ranging from 0 kV to 1 kV. For DNA extraction, the wash step is carried out by applying a voltage between electrodes A and C resulting in EOF of

the wash phase across the monolith. The elution step is then carried out by applying a voltage between electrodes B and D, creating EOF of the elution phase over the monolith (Figure 5.3).

Following elution of DNA into the PCR chamber, thermal cycling was carried out using the Peltier heating system contained within the integrated genetic analyser. PCR products were analysed by removing the contents of the DNA amplification chamber, filtering the sample and analysing the aqueous solution using standard capillary gel electrophoresis protocols (see Chapter 2.5.2).

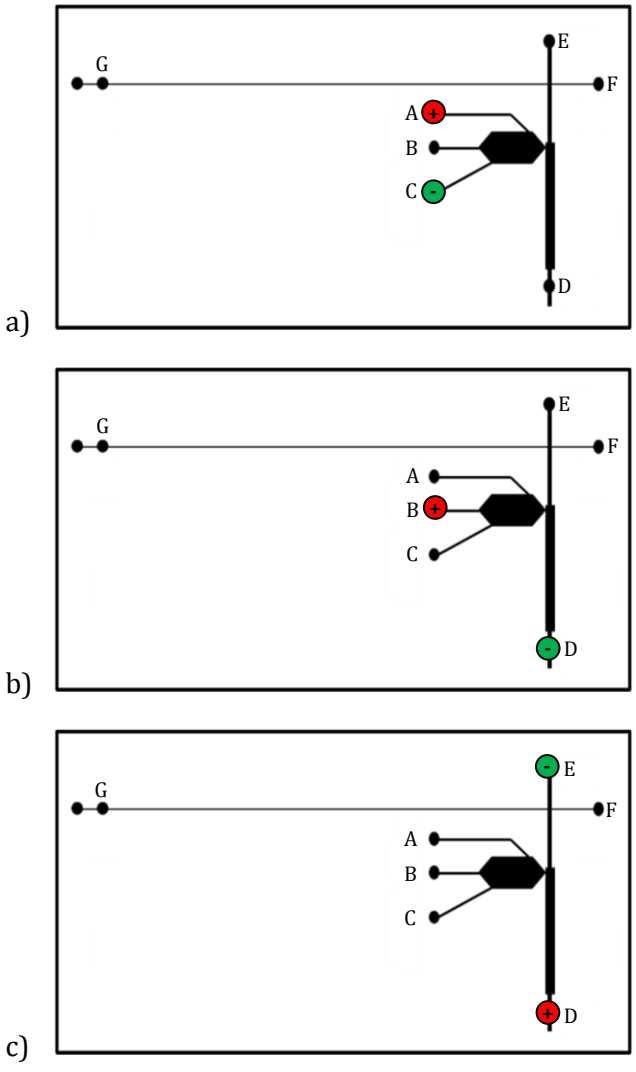


Figure 5.3: Diagram showing the configuration of the electrical connections, on the integrated genetic analysis system, enabling electrokinetic movement within the microfluidic device for the a) wash step, b) elution step and c) reverse shunting step.

5.2.3 Integrated Genetic Analysis Instrument

The integrated DNA extraction and amplification process described above, using electrokinetic movement, was carried out on the genetic analysis instrument developed by Dr. Peter Docker and JLS Designs Ltd. Loading of the microfluidic device, containing the biological sample, all necessary reagents and sealed with carbon electrodes, is a simple procedure which involves placing the device between micromachined posts on the instrument (see Figure 2.12c). Two of the posts are sprung-loaded to provide flexibility in case of size variations during microfluidic device fabrication. Once the lid has been closed, the individual processes can be controlled via the touch-screen monitor (Figure 5.4).

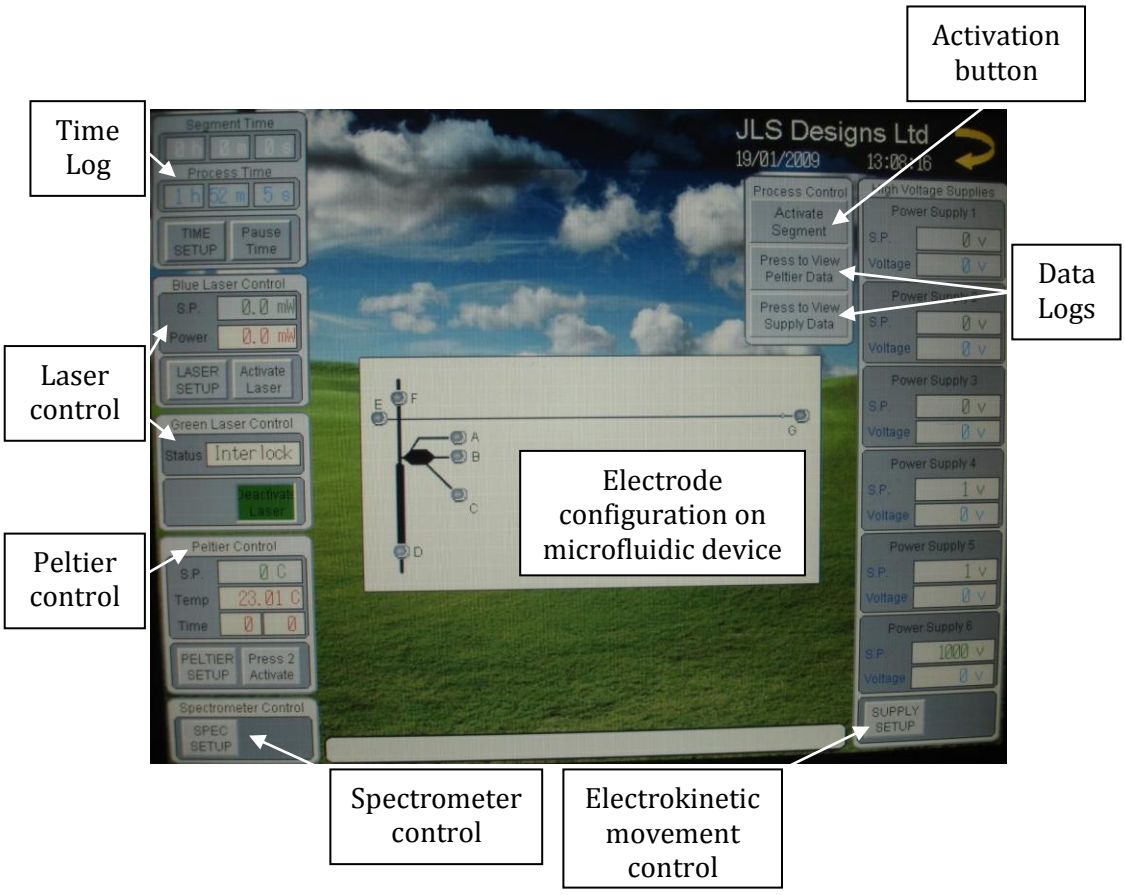


Figure 5.4: Photograph showing the home page of the touch-screen control panel for operation of the integrated genetic analysis system.

The integrated genetic analysis system is also capable of recording voltage and temperature profiles, which enables the user to continually monitor the performance of the system. A recipe editor is also available which can be used to build up a chain of executable commands enabling the entire process of DNA extraction and amplification to be run without operator input once the process has been initiated.

5.3 Results & Discussion

5.3.1 Initial Integrated Genetic Analysis using Hydrodynamic Pumping

Hydrodynamic pumping was demonstrated in Chapter 3 to produce high yields of DNA during the elution phase of the DNA extraction process and so initially it was decided to investigate the use of hydrodynamic pumping for producing an integrated system. Industrial collaborators at the Centre for Integrated Photonics (CIP) [Ipswich, UK] developed a control system which enabled 50 µl syringes containing DNA loading solution, wash solution, DNA elution solution and PCR reagents to be connected to the microfluidic device for hydrodynamic pumping (Figure 5.5).

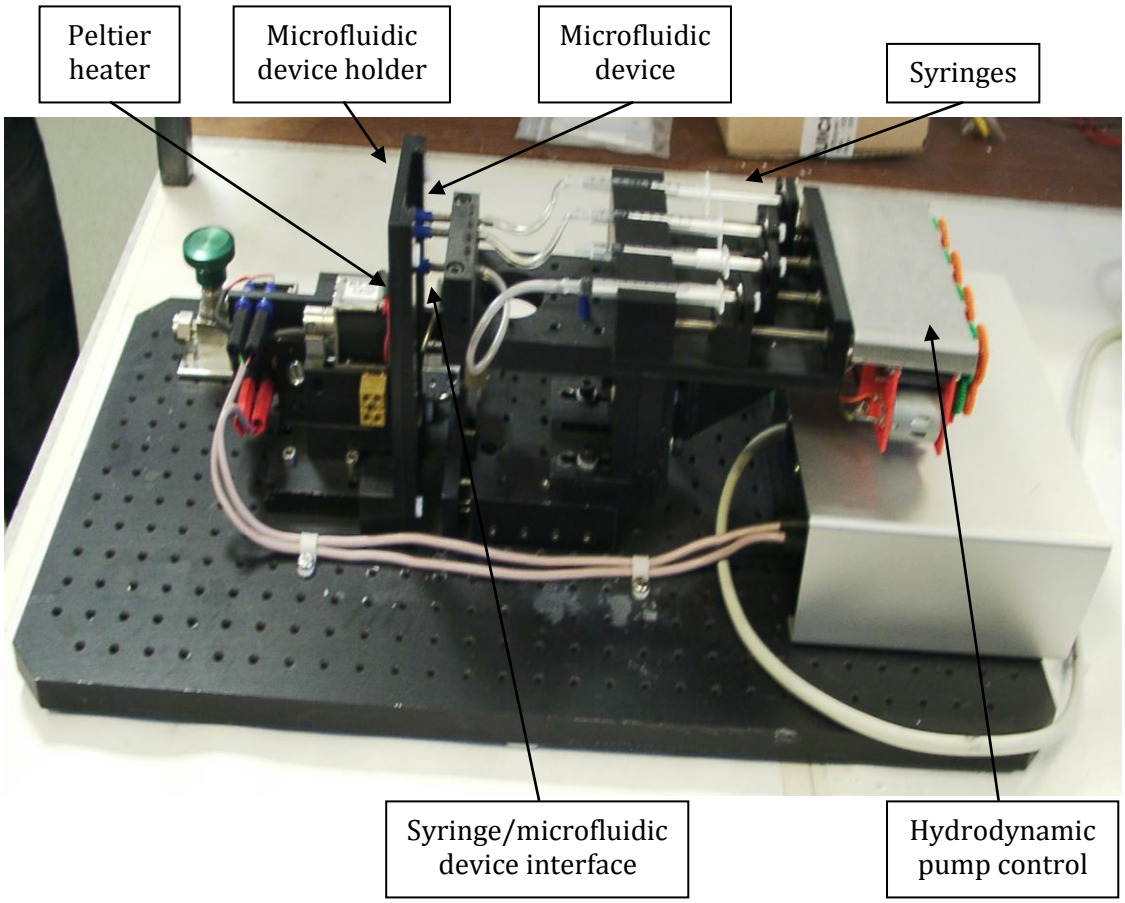


Figure 5.5: Photograph showing hydrodynamically-driven integrated genetic analysis system, developed by CIP. The microfluidic device is loaded vertically into the holder.

After initial testing of hydrodynamic pumping for system integration, a number of problems became apparent. Adsorption of the DNA polymerase enzyme onto the connectors required for attachment of hydrodynamically-driven syringe pumps to the microfluidic device was demonstrated in Chapter 4. However, the most significant problem encountered was the difficulty of creating a completely sealed interface between the syringes and the microfluidic device. Not only was reproducibility almost impossible to achieve, but leaking of solutions creates the potential for contamination, an issue which must be avoided at all cost especially when dealing with forensic samples. When working on individual microfluidic devices in a research setting, tubing can be held securely in position on the microfluidic device by attachment using an epoxy resin. The tubing can then be attached to syringes through the use of connectors, such as MicroTight® Adapters [Upchurch Scientific, UK], creating a sealed interface. However, when looking further ahead to the mass production of microfluidic devices for remote analysis, the attachment of tubing and connectors is not practical. In the system developed by CIP, the use of O-rings and pressure sealing of the interface between syringes and the microfluidic device was examined as this process can be automated. While some degree of success was achieved, i.e. solutions were pumped around the microfluidic device, the difficulty of not being able to reproducibly achieve a completely sealed interface makes this system unworkable. While there is no doubt that hydrodynamic pumping can successfully be used for the application intended here,[190-192] the long-term goals of the project, i.e. to produce a system which can be used remotely and which does not require specialist knowledge to operate, mean that hydrodynamic pumping is unlikely to fulfill these requirements.

For the complete integrated genetic analysis system, the amplified PCR products will need to be electrokinetically injected into a capillary and then subjected to electrophoresis for size separation to occur. As these steps will require the inclusion of electrodes on the microfluidic device and power supplies in the external control system, it would seem logical that if all movement within the microfluidic device could be controlled

electrokinetically then this would make a portable system easier to achieve as it eliminates the need for moving parts. Therefore the compatibility of electrokinetic movement with DNA extraction and amplification was investigated.

5.3.2 DNA Extraction using Electro-osmotic Pumping

As the DNA extraction methodology employed relies on the use of a porous monolith for DNA adsorption, it was hypothesized that this could have a dual functionality by also acting as a pump for EOF preventing reversible flow of solutions. For DNA extraction to be performed using EOP it was essential to determine if all the solutions required could be moved around the microfluidic device using this technique. While it is possible that the binding solution containing DNA could be added manually as there has to be some way of initially loading a sample into the microfluidic device, it is essential that wash and DNA elution steps can be achieved using EOP.

Preliminary studies carried out by Jennifer Oakley (PhD student on the same project developing processes downstream of PCR, i.e. separation and detection, for the complete integrated system) showed that isopropanol, commonly used for the wash step, exhibits poor electro-osmotic movement and so an alternative was sought. The wash solution must be capable of removing cellular and proteinaceous debris whilst maintaining DNA adsorption on the solid-phase. Ethanol has also been reported in the literature for use as the wash solution and it was demonstrated that it can support electro-osmotic movement. In addition, 100 mM sodium chloride (NaCl) was added to the wash gel to facilitate faster EOF and therefore reduce the overall time for DNA extraction.

While it was shown that ethanol could successfully be moved around the microfluidic device, reservoirs of solution were required to ensure that continuous electrical conduct was maintained. The requirement for such reservoirs creates problems with how to store the necessary solution and increases the potential for contamination. In order to enable

storage of reagents on the microfluidic device to produce a self-contained system, the wash solution was encapsulated in LMT agarose gel. To keep gel concentrations consistent throughout the device, a 1.5% gel was used as this was found to be optimum for PCR (see Chapter 5.3.3). A range of different percentage ethanol solutions were tested to examine the solubility of agarose at a concentration of 1.5%. It was found that the solubility limited production to a 50% (v/v) ethanol gel-based solution. Analysis of the wash fraction, using PicoGreen®, showed that despite the lower alcohol percentage no substantial DNA loss from the solid-phase occurred during the wash step. By applying a range of voltages, across a monolith, to a 50% (v/v) ethanol gel solution contained within a channel the flow rate resulting from EOP was measured. Figure 5.6 shows an example of EOP for washing of the monolith.

During the DNA elution phase, a low ionic strength buffer is used to facilitate desorption of DNA from the silica solid-phase. Previous work on hydrodynamic pumping used 10 mM TE buffer for elution and it was found that this solution fully supported electro-osmotic movement. An elution gel was produced consisting of 10 mM TE buffer in 1.5% (w/v) LMT agarose. Figure 5.7 shows an example of EOP for elution of DNA from the monolith, visualised using a neutral rhodamine B dye [Sigma-Aldrich, UK]. Increasing the concentration of TE buffer enabled faster flow rates to be achieved but higher concentrations of EDTA in the buffer act to chelate the Mg^{2+} ions required as co-factors for the DNA polymerase and so can reduce the efficiency or even inhibit the DNA amplification reaction.

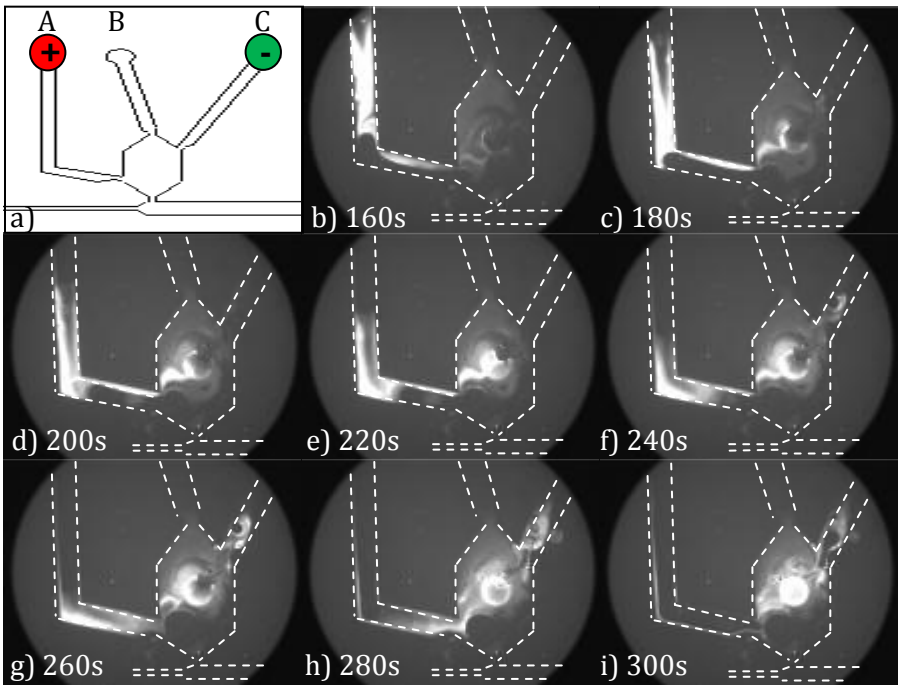


Figure 5.6: Movement of wash gel across monolith using EOP: a) schematic depicting microfluidic device geometry and electrode orientation, b) to i) fluorescence microscopy images showing the movement of a 50% ethanol gel, using EOP, for the wash of the solid-phase matrix. A voltage of 100 V/cm was applied between electrodes A and C resulting in an average flow-rate of 0.6 µl/min (n=3).

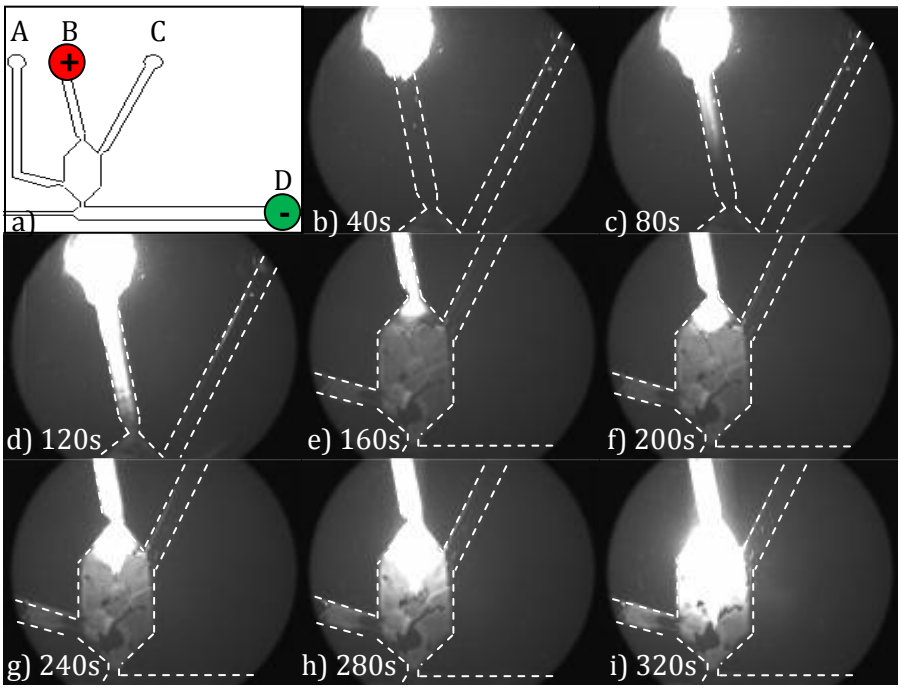


Figure 5.7: Movement of elution gel across monolith using EOP: a) schematic depicting microfluidic device geometry and electrode orientation, b) to i) fluorescence microscopy images showing the movement of a 10 mM TE buffer gel, using EOP, for the elution of DNA from the solid-phase matrix. A voltage of 100 V/cm was applied between electrodes B and D resulting in an average flow-rate of 0.4 µl/min (n=3).

While the flow rates achieved using EOP were demonstrated to be less than that for hydrodynamic pumping, the slower flow rates will theoretically allow more time for the reagents to come into contact with the solid-phase facilitating DNA extraction. Once the potential flow rates had been established, the effects of time and voltage on DNA extraction efficiency were investigated. Microfluidic devices were prepared according to the methodology given in Chapter 5.2.2. DNA in GuHCl binding solution was then manually injected onto the monolith. The wash and elution steps were then carried out by applying a range of voltages (75 – 175 V/cm) for different lengths of time (5 – 15 minutes). The eluted samples were collected and analysed by PicoGreen® to enable DNA

quantification. Maximum DNA yields during the elution step were produced when a voltage of 125 V/cm was applied for 15 minutes (Figure 5.8).

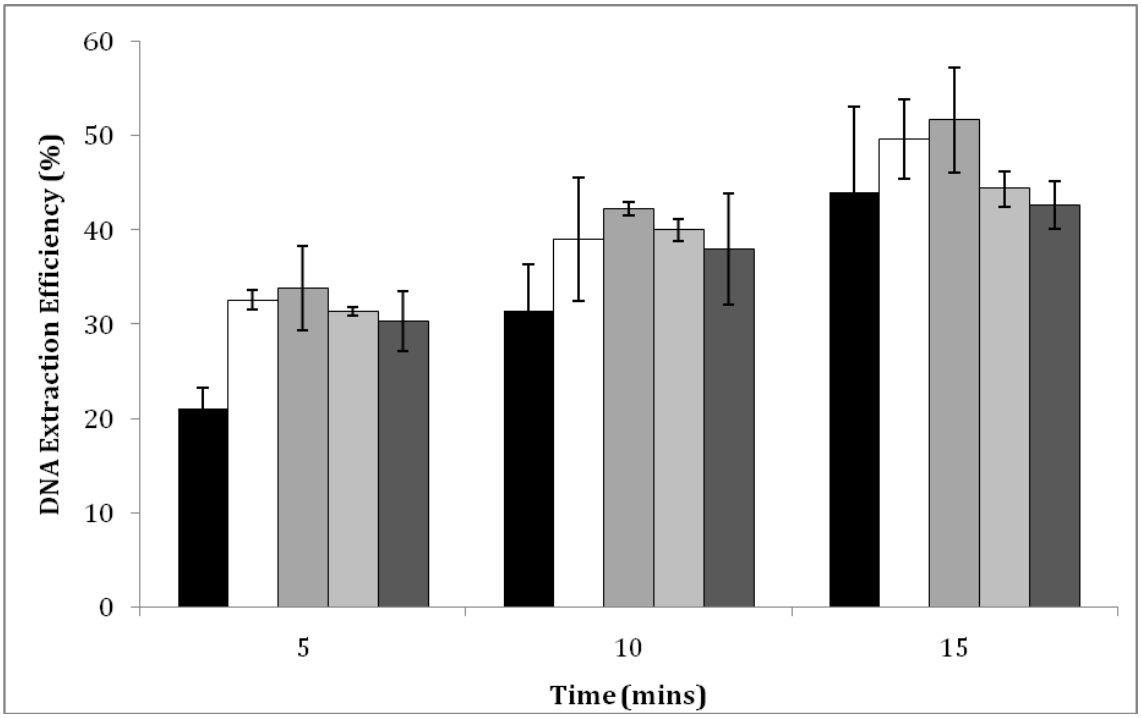


Figure 5.8: Graph showing the effect of time and voltage on DNA extraction efficiency. A range of voltages were tested: 75 V/cm (■), 100 V/cm (□), 125 V/cm (▩), 150 V/cm (▨) and 175 V/cm (▩) (n=3).

Initially, using a higher applied voltage (from 75 to 125 V/cm) increases the DNA yield during the elution step as the flow rate of EOP is higher so a greater volume of TE buffer will be flowed over the monolith releasing more of the DNA. However, when voltages greater than 125 V/cm were applied, the electrophoretic movement of the DNA, in the opposite direction to the electro-osmotic flow, has a greater influence on the DNA and so more DNA is pulled in the opposite direction reducing the extraction efficiency.

The apparent mobility of the DNA (μ_{app}) is given by the following equation:

$$\mu_{app} = \mu_{eof} + \mu_{ep} \quad \textbf{(Equation 5.1)}$$

where μ_{eof} is the mobility due to electro-osmotic flow and μ_{ep} is the electrophoretic mobility. The mobility due to electro-osmotic flow, μ_{eof}, is calculated by:

$$\mu_{eof} = \frac{\nu_{eof}}{E} \quad \textbf{(Equation 5.2)}$$

where ν_{eof} is the electro-osmotic flow velocity (cm/second) and E is the applied electric field (V/cm). The μ_{eof} values for 50% ethanol and 10 mM TE buffer gels were calculated based on flow velocities of 0.6 µl/min and 0.4 µl/min, respectively, using an applied electric field of 100 V/cm. This resulted in a μ_{eof} value of +2.86 x 10^{-5} cm^2/V/sec for the wash gel and a μ_{eof} value of +1.9 x 10^{-5} cm^2/V/sec for the elution gel.

As the reaction is carried out in a gel-based environment the electrophoretic mobility is given by:

$$\log \mu_{ep} = \log \mu_o - K_R T \quad \textbf{(Equation 5.3)}$$

where μ_o is the electrophoretic mobility in free solution, K_R is the retardation coefficient and T is the gel concentration. K_R is dependent upon the size of the DNA fragments being subjected to electrophoresis. During DNA extraction, shearing forces result in DNA fragments of tens of kilobasepairs (kbp).[217] As a conservative estimate a K_R value for a 12 kbp fragment of DNA in a 1.5% agarose gel was used.[218] The electrophoretic mobility in free solution is -3.75 x 10^{-4} cm^2/V/sec with a gel concentration of 1.5% agarose used throughout the system.[165] Therefore μ_{ep} can be calculated as -5.01 x 10^{-8} cm^2/V/sec.

Therefore the apparent mobility of the DNA (Equation 5.1) during the wash and elution phases of DNA extraction procedure is +2.85 x 10^{-5} cm^2/V/sec and +1.89 x 10^{-5} cm^2/V/sec, respectively. While these values are only approximate, due to the exact size of the DNA

fragments being unknown, EOP represents the dominant force resulting in bulk movement of the DNA towards the cathode.

Increasing the amount of time EOP is carried out for increases the amount of DNA yielded during the elution step and therefore the efficiency of the DNA extraction process. The longer the TE buffer is pumped over the monolith, the more time there is for the DNA to be desorbed and transferred into the DNA amplification chamber. It was hypothesised that further increasing the length of the elution step would result in higher DNA yields, but in the development of a portable device time is a crucial issue. Therefore, a DNA extraction efficiency of 52%, achieved using an elution voltage of 125 V for 15 minutes (Figure 5.8), was deemed as sufficient to enable a compromise with speed of analysis.

DNA extraction using EOP on the microfluidic device was also carried out using biological samples direct from a buccal swab. Once the buccal swab had been scraped along the inside of the cheek of a volunteer it was immersed in 5 M GuHCl solution. A 20 µl aliquot of this solution was then manually injected onto the microfluidic device through the port above the monolith. Any waste DNA binding solution was collected in channel C and the DNA extraction procedure carried out as above. DNA yields of 0.44 ± 0.07 ng/µl were produced from the elution of DNA from the monolith using EOP.

As the isopropanol used for the wash step during hydrodynamic pumping had to be replaced with ethanol for EOP, it was considered necessary to carry out a direct comparison between the two pumping methodologies. Therefore DNA extraction using the hydrodynamic pumping methodology, as described in Chapter 3, was repeated but this time ethanol was used as the wash solution. The average DNA extraction efficiency recorded was found to be 72%, which represents a 15% decrease compared to when isopropanol was used. The discrepancy between the results may be due to the relative solubility of DNA in the two different alcohols. Isopropanol (dielectric constant (k) = 24.3) is a less polar solvent than ethanol (k = 18.3) and therefore DNA will be less soluble in the

isopropanol and less likely to be removed from the monolith during the wash phase of the DNA extraction procedure.

In forensic casework it is important that a portion of the DNA sample is retained so that, if required, it can be re-evaluated either by the opposing council or if new technology becomes available that would reveal additional information. Using EOP, a significant amount of DNA is retained on the monolith after the elution step which could then be maintained in storage. If required, it is possible that in a laboratory setting hydrodynamic pumping could be applied and additional DNA eluted for subsequent testing purposes.

5.3.3 DNA Amplification in Agarose Gel

In order to be able to store PCR reagents on the microfluidic device there needs to be a protective mechanism in place as the *Taq* DNA polymerase enzyme in particular is temperature sensitive and usually requires storage at -20°C. A number of commercial companies have developed ways of producing stable mixtures containing multiple PCR reagents combined together, which in addition reduces the number of pipetting steps required to perform DNA amplification. One example of this is so-called 'PCR beads', for example Illustra PuRe Taq Ready-To-Go™ PCR beads [GE Healthcare, UK], which contain all the necessary reagents (minus the specific primers for the loci under investigation and the sample DNA) encapsulated within agarose.[219] The reaction-specific primers and DNA are added immediately prior to the amplification reaction taking place. As the number of preparatory steps required is reduced there is less risk of pipetting errors and a reduced risk of contamination. In addition, such 'PCR beads' are usually stored at 4°C rather than the -20°C usually required for the DNA polymerase.

Production of in-house 'PCR beads' was carried out, which contained all the necessary reagents for DNA amplification minus the sample DNA. An LMT agarose was used as PCR reagents could be added to the molten gel solution at a lower temperature, minimising any

problems associated with thermal damage, and also to ensure that the gel used for encapsulation would be molten at the temperatures required for PCR enabling sufficient mixing of reagents.

The use of a Hot-Start DNA polymerase can also help to maintain DNA amplification efficiency during the production of the 'PCR beads'. During the preparation of the PCR reagent mixture, non-specific annealing can occur between primers and template sequences that are not entirely complementary or between different primer sequences. Standard DNA polymerases exhibit some activity at the lower temperatures required for PCR preparation and so amplification of these non-specific products can occur. In order to encapsulate the PCR reagents in the agarose gel, they must be added during the molten phase and so there is potential for generation of non-specific PCR products at the required temperatures. In order to prevent this a range of Hot-Start DNA polymerases, namely AmpliTaq™ Gold [Applied Biosystems, UK], FastStart *Taq* [Roche, UK] and GoTaq® [Promega, UK] were evaluated.

Each of the different Hot-Start DNA polymerases was included in a standard PCR reagent mixture (Table 2.1) produced in a gel-base format as described in Chapter 5.2.2. DNA amplification on the microfluidic device was then carried out for 28 cycles at 94°C, 60°C and 72°C, with 30 second hold-times at each temperature. An initial denaturation step was included, the length of which varied depending on DNA polymerase used (see Table 2.2 for details). PCR products were analysed using conventional capillary gel electrophoresis.

Whilst the AmpliTaq™ Gold DNA polymerase was shown to work well for multiplex PCR analysis, as demonstrated by the inclusion in the SGMplus™ DNA profiling kit, the activation time is 11 minutes which is not compatible with the increased speed of analysis required from an integrated genetic analysis system. Both FastStart *Taq* and GoTaq® DNA polymerase require only a 2 minute activation step therefore reducing the amount of time necessary. GoTaq® DNA polymerase was found to produce better results than FastStart

Taq DNA polymerase, in terms of reproducibility and signal intensity, and so was used in all further experiments. Both AmpliTaq™ Gold and FastStart *Taq* are chemically modified DNA polymerase enzymes that contain heat labile blocking groups preventing enzyme activity prior to their removal at DNA denaturing temperatures.[2, 220] Whereas a proprietary antibody is used to block GoTaq® DNA polymerase activity until the initial Hot-Start step.[221] It is possible that this difference in the production of Hot-Start activity may account of the increased performance of the GoTaq® DNA polymerase for this application.

The effects of agarose gel concentration on the efficiency of the DNA amplification reaction were investigated. All PCR reagents (see Table 2.1), including TH01 primers, were encapsulated in agarose gel solutions of varying concentrations, up to 2.5% (w/v). Once DNA had been added the samples were subject to PCR amplification on a standard thermal cycler (N.B. Amplification was performed on a standard thermal cycler rather than the microfluidic system to enable higher throughput of multiple samples). The amplified samples were then analysed using capillary gel electrophoresis and the peak heights of the two alleles at the TH01 locus recorded, providing a measure of relative fluorescence intensity. An agarose gel concentration of 1.5% was chosen as the optimum for producing in-house 'PCR beads' (Figure 5.9).

A sufficient gel concentration is required to support the PCR reagents for long-term storage but it must not be so high that it leads to inhibition or reduced efficiency of the amplification reaction. A significant decrease in fluorescent signal intensity was observed when agarose gel concentrations of 2% or more were used, thus preventing their use for maximum PCR performance. There was no significant difference in the fluorescent signal intensity for agarose gel concentrations up to 1.5%, therefore this maximum was chosen as it will provide the greatest degree of structural integrity protecting the encapsulated PCR reagents.

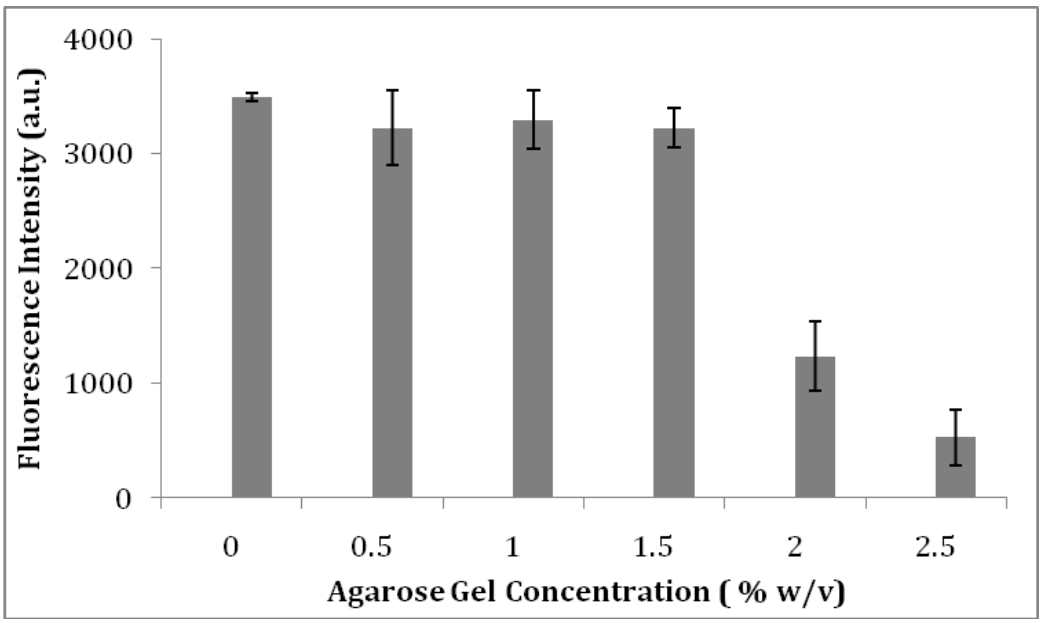


Figure 5.9: Effect of agarose gel concentration on DNA amplification efficiency. PCR was carried out in the presence of 0.5% to 2.5% (w/v) LMT agarose gel. An aqueous positive control was used to provide a comparison (n=3).

Once the optimum agarose gel concentration had been established the effects of encapsulation of the PCR reagents was investigated with regard to their long-term stability. All PCR reagents, including TH01 primers, were encapsulated in a 1.5% agarose gel solution and these 'PCR beads' were then stored at either room temperature, 4°C or -20°C for up to 8 weeks. All samples were protected from light to avoid photo-bleaching of the fluorophores present on the forward primers which are required for detection. An aliquot of DNA was added to the 'PCR beads' immediately prior to analysis. Following thermal cycling the PCR products were then analysed using capillary gel electrophoresis and the peak heights of the two alleles at the TH01 locus recorded, providing a measure of relative fluorescent intensity (Figure 5.10).

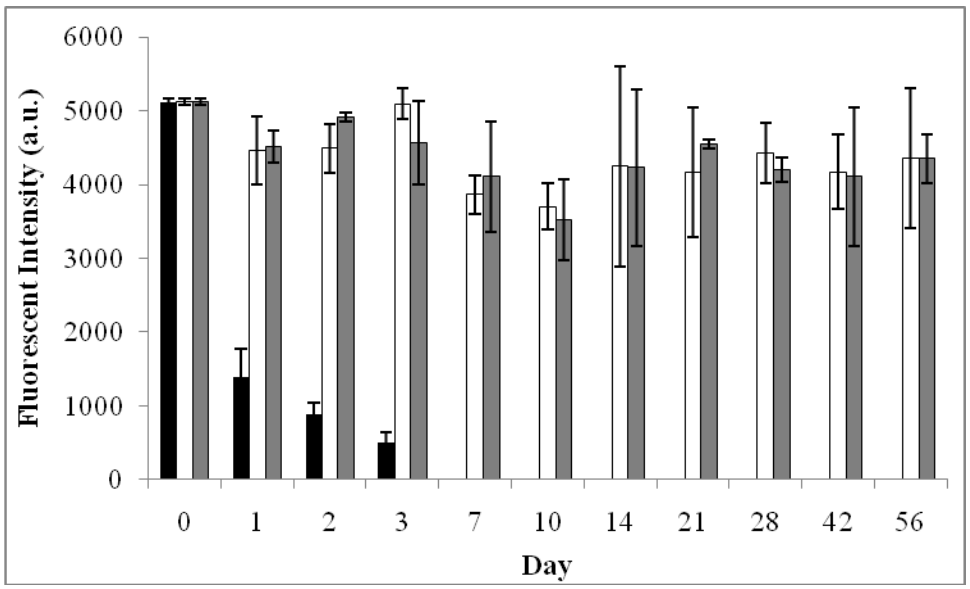


Figure 5.10: Stability study of agarose gel-encapsulated PCR reagents. All PCR reagents, including TH01 primers, were encapsulated in a 1.5% agarose gel solution and stored at either room temperature (■), 4°C (□) or -20°C (▩) for up to 8 weeks (n=3).

Those PCR reagents which were stored at room temperature exhibited significant reduction in fluorescent signal intensity after just 24 hours and by Day 7 no PCR products were detectable. The lack of PCR product is most likely due to loss of activity of the DNA polymerase enzyme at the elevated temperature. PCR reagents which were stored at either 4°C or -20°C showed no significant loss of fluorescent signal intensity even after 8 weeks of storage.

The ability to store PCR reagents on the microfluidic device has a number of distinct advantages. Portability of the system can be increased if the necessary reagents can be stored at 4°C rather than -20°C as this temperature is much easier to achieve remotely, for example through the use of a cool box for 'at scene of crime' use or storage in the fridge at a custody suite. The storage of reagents within the microfluidic device not only drastically reduces the input required from the end-user but also reduces the risk of contamination as

the only external component required to enter the device is the DNA sample. Eliminating the need for multiple pipetting steps in producing the PCR reagent mixture for addition to the microfluidic device also reduces the likelihood of volume errors which may result in an suboptimal or inhibited DNA amplification reaction.

The few papers previously published in the literature on storage of PCR reagents on microfluidic devices have focussed solely on freeze-drying of the reagents.[222, 223] While successful amplification could be achieved following long-term storage at -20°C, DNA polymerase activity was reduced to 50% when the freeze-dried PCR reagents were stored at 4°C.[222] Although the freeze-drying process has been demonstrated to offer a degree of protection to the PCR reagents, it is a time-consuming, multistep process which can result in decreased DNA polymerase activity.[223] The use of agarose gels to encapsulate the PCR reagents rather than freeze-dry them enables storage of the microfluidic device at higher temperatures, specifically 4°C.

A direct comparison of the amplification efficiency of a single STR locus (TH01) was carried out in aqueous and gel-based environments. PCR reagents in aqueous or 1.5% agarose gel solutions, had DNA added and were subject to amplification either using both 0.2 ml polypropylene tubes in a conventional PCR instrument and a microfluidic devices in the integrated genetic analyser. Following thermal cycling the samples were then analysed using capillary gel electrophoresis and the peak heights of the two alleles at the TH01 locus recorded, providing a measure of relative fluorescent intensity (Figure 5.11).

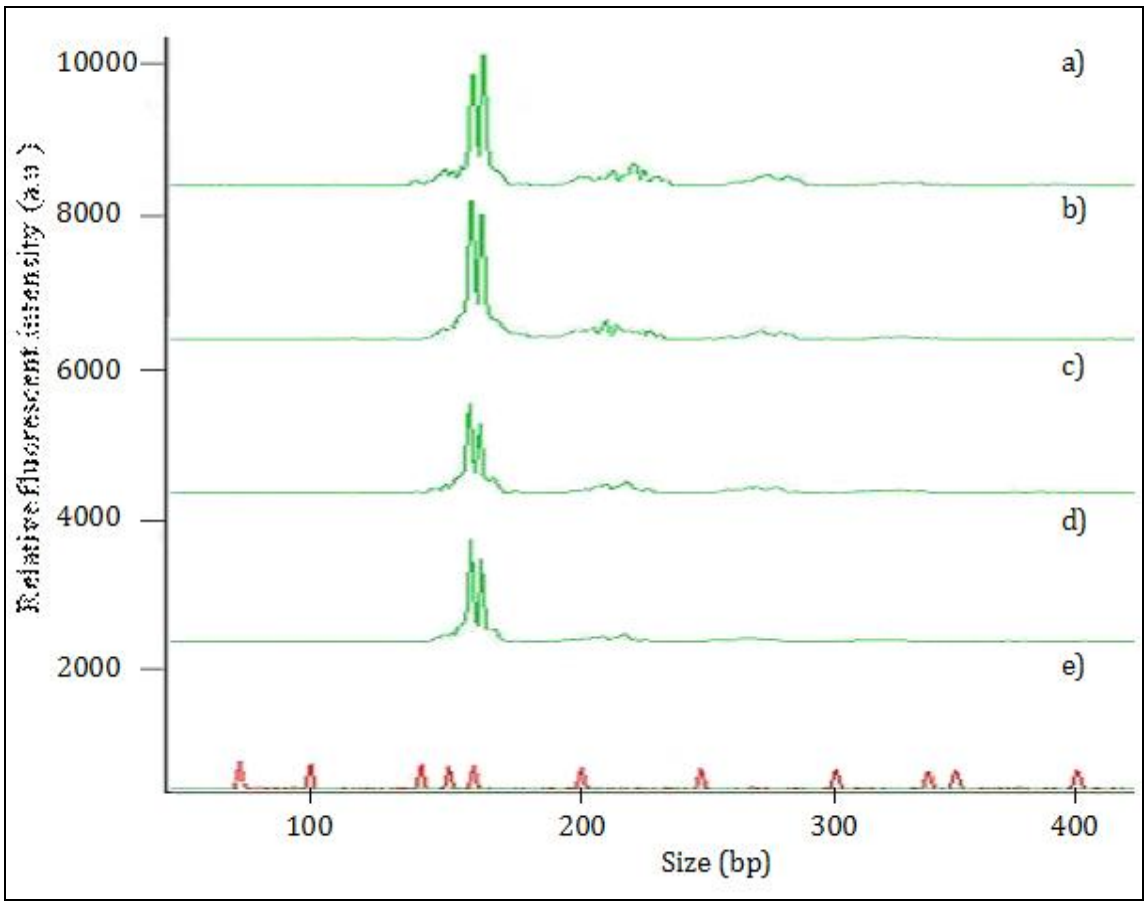


Figure 5.11: Electropherograms produced from PCR products amplified under different conditions: a) aqueous sample amplified on conventional PCR instrument; b) gel-based sample amplified on conventional PCR instrument; c) aqueous sample amplified on the microfluidic device using Peltier heating in the integrated genetic analyser; d) gel-based sample amplified on the microfluidic device using Peltier heating in the integrated genetic analyser; e) negative no DNA control ran on the conventional PCR instrument.

No significant differences in signal intensity were observed between the control samples, as expected from previous results during the gel-based PCR optimisation process. When DNA amplification was carried out on the microfluidic device, the difference in amplification efficiency between the aqueous and gel-based systems was more pronounced. The relative fluorescence intensity of the PCR products produced in an

aqueous environment on the microfluidic device was only 65% of the positive control sample, whereas the PCR products produced in gel-based environment were 72% of their control sample. Therefore, in addition to the advantages brought about by storage of reagents on the microfluidic device, the use of a gel-based matrix for DNA amplification within the microfluidic environment increases the efficiency of the reaction. This offers the possibility of being able to perform fewer PCR cycles, whilst still achieving the same fluorescent signal intensity, which ultimately can result in a valuable time-saving for the entire DNA analysis process.

Once optimisation of the agarose gel environment had been established using a single locus, the next step was to transfer the multiplex reaction (Chapter 4.3.4) into the same environment. Successful amplification of all nine multiplexed loci (8 STR markers plus the Amelogenin sex marker) was achieved in a 1.5% LMT agarose gel solution on the integrated genetic analysis system, although it was observed that the DNA profiles produced were not as balanced as the aqueous format. It may be possible that the gel-based format has an effect on the mobility of the diffusion of larger DNA molecules during the amplification reaction limiting their ability to completely interact.

5.3.4 Integration of DNA Extraction by EOP and DNA Amplification

Once the individual DNA extraction and amplification techniques had been optimised for use on the microfluidic device in an electrokinetically-driven, enclosed gel-based format, they were combined into a single integrated system. The success of the combined DNA extraction and amplification process was assessed by analysing the sample following DNA amplification using standard capillary gel electrophoresis.

The electrokinetic movement required to perform DNA extraction within the microfluidic device causes a certain degree of Joule heating to occur. In particular, the elution of the DNA from the thermally activated silica monolith can lead to the premature heating of the

PCR reagents which have been pre-loaded into the DNA amplification chamber. As a precaution the Peltier heater, usually used to provide thermal cycling for DNA amplification, was set and held at 4°C during the DNA extraction process to prevent heating of the PCR reagents during this phase.

When the DNA extraction phase was initially performed using the optimum conditions described in Chapter 5.3.2, i.e. DNA elution at 125 V/cm for 15 minutes, the results of a single locus DNA amplification reaction was very poor. Decreasing the applied voltage down to 100 V/cm for the DNA elution step resulted in a more efficient downstream amplification process. It is believed that when the higher voltage is applied some of the other charged species required for PCR, such as Mg^{2+} ions or the primers pre-loaded onto the device in a gel format, will also migrate and therefore their distribution within the PCR chamber may not be homogenous to begin with. Although it is understood that homogeneity is created during thermal cycling due to the convection currents created, the first few cycles of PCR are crucial and so if conditions are not optimum from the beginning of the reaction this can cause problems.

Despite the success of the integrated DNA extraction and amplification process for PCR of a single locus, it was not possible to successfully directly apply the same EOP conditions to DNA extraction integrated with multiplex DNA amplification. To investigate the possible migration of other PCR components further, a reverse shunting step was used following the DNA elution step. This involved applying a 100 V/cm voltage from electrodes D to E pulling any molecules which have migrated completely towards electrode D to be moved back towards the DNA amplification chamber. A range of reverse shunting times, from 5 to 60 seconds were investigated, yielding a different number of amplified loci depending upon the time the voltage was applied for. Optimum results were obtained when a 5 second reverse shunting step was used, resulting in successful amplification of all nine loci.

To ensure that the integrated electrokinetically-driven system developed was suitable for use in a custody suite setting, DNA extraction and amplification from buccal swabs was performed. The results in Chapter 5.3.2 showed that DNA could be extracted from buccal swabs using EOP on the microfluidic device with average yields of 0.44 ng/µl. Once the DNA had been transferred by EOP into the DNA amplification chamber, containing preloaded PCR reagents, thermal cycling was performed. Successful PCR of the previously reported multiplex was achieved, showing amplification of all nine loci (Figure 5.12).

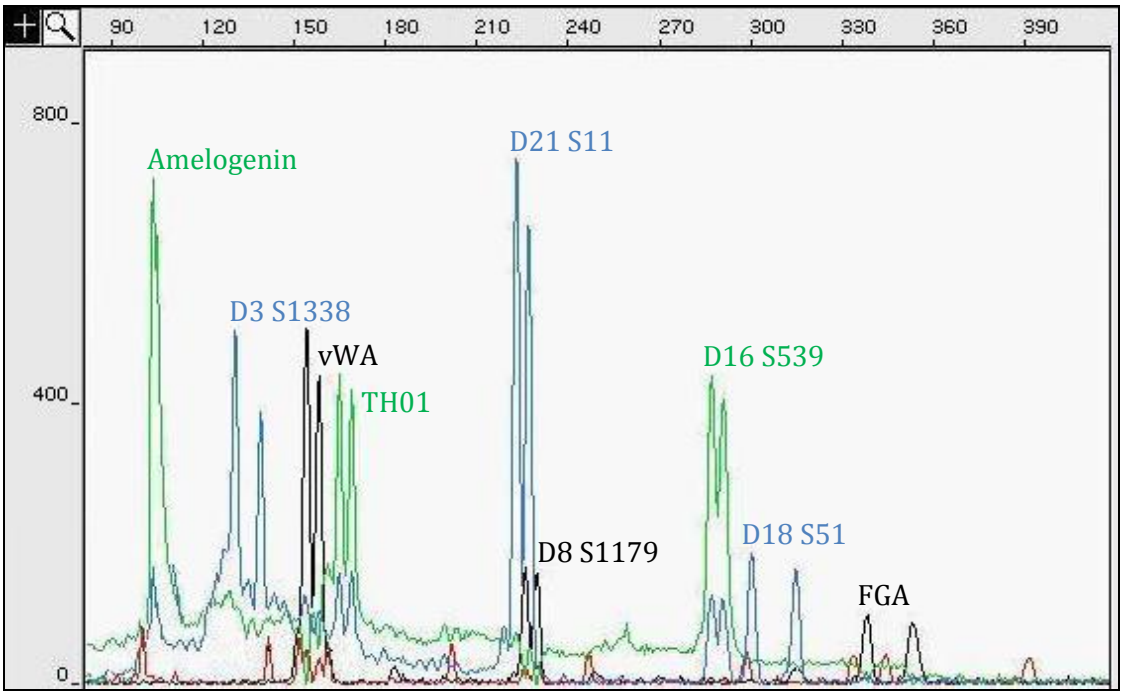


Figure 5.12: Example electropherogram showing 9-plex PCR DNA amplification products in an agarose gel-based format on a microfluidic device using Peltier heating within the integrated genetic analysis system. Size (base pairs) is shown on the x axis and fluorescent intensity (arbitrary units) is shown on the y axis. Following a 2 minute initial denaturation step, for activation of the GoTaq® DNA polymerase, 35 thermal cycles were performed at 94°C, 60°C and 72°C, with hold-times of 30 seconds at each temperature.

5.4 Summary

A novel integrated system has been reported for DNA extraction and amplification on a single microfluidic device. Evaluation of a hydrodynamically-driven integrated system highlighted problems with creating a sealed liquid interface, to enable movement of reagents around the microfluidic device, in a reproducible and practical manner. In light of this an alternative pumping mechanism was sought and an electrokinetically-driven system proved successful.

While the DNA extraction efficiency using EOP was not as high as that for hydrodynamic pumping (52% compared to 72%) the amount of DNA recovered was of sufficient quantity and quality for successful DNA amplification. In addition there are several advantages provided by the electrokinetically-driven system including easier mechanical connection to the microfluidic device, elimination of the need for 'off-chip' pumps and negligible pressure drops induced by fluid movement through the channels of the microfluidic device.[165]

While there are few reported cases in the literature of integrated DNA extraction and amplification, those which there are rely on the use of hydrodynamic pumping to combine the flow of eluted DNA with a double concentrated PCR reagent solution.[190-192] Despite the demonstrated functionality of this technique, it relies on a concentrated PCR solution which increases the cost of reagents per analysis, which is counterintuitive in a microfluidic system as compared to the method presented here which uses a standard PCR reagent solution.

The ability to store all the necessary reagents on the microfluidic device for performing nucleic acid-based reactions has received little attention in the literature. Freeze-drying of PCR reagents onto the internal surface of the PCR chamber on a microfluidic device enabled successful DNA amplification to be performed after six months storage at -20°C.[222] When the PCR reagents were stored at higher temperatures, 4°C or room temperature,

however, the DNA polymerase was found to be half or completely inactive at the respective temperatures. While freeze drying offers a degree of protection to the PCR reagents as compared with low temperature storage, it is a time-consuming, multistep process which has been reported to decrease DNA polymerase activity.[223] The encapsulation of PCR reagents within agarose gel to enable storage on the microfluidic device as demonstrated here provides an alternative mechanism to previously reported freeze drying methodology and enables storage of the device at 4°C, for at least 8 weeks, without loss of DNA polymerase activity.

In addition, the use of a gel-based system offers advantages when dealing with air bubbles. The presence of bubbles in microfluidic systems represents a major issue as it can result in failure of electrokinetic movement and/or PCR. Air bubbles can cause breaks in the electrical circuit preventing movement by EOP or failure of PCR due to localised temperature differences of up to 5°C.[117, 181, 224] By carrying out EOP and PCR in a completely agarose gel-based environment bubble formation is minimised and EOP can still occur even in the presence of bubbles.

6 Conclusions

The work presented in this thesis details an evaluation of DNA extraction and amplification performed on a microfluidic device. Optimisation of both techniques was carried out using hydrodynamic and electrokinetic pumping. A prototype control system was simultaneously developed as part of a more extensive programme of work forming a collaborative effort between Dr. Peter Docker and JLS Designs Ltd, which was evaluated as part of the integrated microfluidic device development.

Solid-phase DNA extraction was performed on the microfluidic device using silica-based methodology, which allows elution of the DNA in a PCR compatible format, facilitating the direct integration of downstream DNA amplification methodology. In addition, solid-phase extraction allows pre-concentration of DNA from the biological sample, important when dealing with limited sample types as may be the case in a forensic setting. A comparison was made of three different types of silica-based solid-phases, silica beads, thermally activated silica monoliths and photo-initiated silica monoliths, to determine the most suitable for inclusion in the microfluidic device. Evaluation of the DNA extraction efficiencies (see Equation 2.1) of the different solid-phases showed that the thermally activated silica monolith exhibited superior performance (82% ± 6%) compared to the use of silica beads (76% ± 32%) or photo-initiated monoliths (23% ± 5%). The use of thermally activated silica monoliths also offered advantages over the other solid-phases in terms of reproducibility.

It was found that the thermally activated silica monoliths were relatively easy to produce, made of just potassium silicate and formamide, and cured at 90°C overnight. Precision placement of the monolith within the microfluidic device was achieved by exploiting DNA extraction chamber geometry and using glycerol as an added containment measure (see Figure 3.13). DNA was successfully extracted from a range of biological sample types, namely buccal cells, saliva, whole and dried blood, using these monoliths in a

hydrodynamically-driven system. For all sample types the eluted DNA was of sufficient quantity and quality for successful PCR DNA amplification to take place.

Carrier molecules, such as RNA, have previously been reported to increase DNA extraction efficiency in commercial DNA extraction kits.[53] An evaluation of the compatibility of using carrier RNA molecules in microfluidic DNA extraction systems was performed. It was demonstrated that the inclusion of carrier RNA, in with the GuHCl binding solution, markedly increases DNA extraction efficiency on thermally activated silica-based monoliths in a microfluidic device, confirming that this methodology is suitable for microfluidic applications. No adverse effects on downstream applications such as PCR were observed to be associated with the inclusion of carrier RNA. If required, carrier RNA can therefore be used to increase the recovery of DNA during the extraction procedure in cases where the initial amount of DNA is limited or large amount of DNA are required for downstream processes.

A major issue when performing DNA amplification in a microfluidic system is that the increased surface area to volume ratio can result in DNA polymerase adsorption to the internal surfaces and therefore inhibition of PCR.[122] Depending upon the etch depth of the microfluidic devices used, inhibition of the DNA amplification reaction was predicted to occur (see Figure 4.4). As a result dynamic passivation, using 0.2 µg/µl BSA, 0.01% (w/v) PVP and 0.1% (v/v) Tween-20, and static passivation by TPS silanisation were required. TPS was chosen as it demonstrated the most stability during thermal cycling compared to other silanisation reagents which were shown to suffer from degradation.

A Peltier heater was incorporated into the integrated genetic analysis system to enable thermal cycling for DNA amplification. The system was capable of producing heating and cooling rates of 1°C/second with steady-state holds of ± 0.1°C, which are comparable to conventional PCR instruments. Multiplex amplification of nine forensically relevant loci, common to both UK and US DNA profiling kits, was possible using this system. DNA

amplification using the Peltier heating system also enabled real-time monitoring of the reaction to be carried out, using either an intercalating dye or TaqMan™ style probes.

The use of a custom-made microwave heating system was also evaluated for DNA amplification in a microfluidic device. Successful amplification of the Amelogenin locus from both male and female samples was achieved. Heating and cooling rates, of 65°C/second and 58°C/second respectively, were possible meaning that all temperature transitions for PCR were achievable in less than a second. In addition the precision of the hold at each temperature was also an improvement on conventional thermal cyclers, with steady-state values of ± 0.05°C. The use of microwave heating offers the potential to achieve thermal cycling for PCR in a fraction of the time of conventional PCR instruments, with also a fraction of the PCR reagent volume. Presently, DNA amplification has been demonstrated in volumes as low as 1.1 µl. The suitability of the surface passivation coatings presented here would suggest that even lower PCR reagent volumes are possible within the microfluidic system presented here, but there remains the issue of the 'world-to-chip' interface where there is a practical limitation to the volume which can be introduced into the microfluidic device.

Despite initial successes with hydrodynamic pumping of reagents around the microfluidic device, it became apparent that production of a reproducibly sealed interface between the syringe pumps and microfluidic devices would be very difficult to achieve. If a sealed interface cannot be produced then this creates the potential for leakage of solutions and contamination, a major issue when dealing with forensic samples. The production here of a system which is entirely electrokinetically-driven enables all movement for solutions DNA extraction by EOP, resulting in elution of purified DNA from the monolith surface. In addition, the downstream processes of electrokinetic injection of PCR products and separation by electrophoresis can be performed using this methodology. The use of injection molded carbon-containing polystyrene electrodes not only provides an electrical

contact between the microfluidic device and integrated genetic analysis operating system but also serves to seal the microfluidic device.

The storage of all the necessary reagents for genetic analysis on the microfluidic device is a significant step forward in the development of a truly portable system. Encapsulation of the PCR reagents in a LMT agarose gel confers a degree of protection to the reagents, most likely via the maintenance of structural integrity, enabling them to be stored on the microfluidic device for at least eight weeks when kept at 4°C. This could prove beneficial in producing a microfluidic device which can be taken directly 'off-the-shelf' and transferred onto the integrated genetic analyser, with only the addition of the biological sample required. Advantages include savings in the amount of preparation time, decreased risk of contamination and reduced operator input which could enable the system to be used by non-scientific personal, such as police officers in a custody suite setting.

A number of commercially available miniaturised thermal cyclers have now reached the market, for example, SmartCycler® by Cepheid which is capable of carrying out 96 independently controlled, real-time monitored, DNA PCR amplification reactions in 20 - 40 minutes.[225] The SmartCycler® was developed from the fundamental aspects of DNA amplification miniaturisation shown by the miniature analytical thermal cycling instrument (MATCI) and the second generation advanced nucleic acid analyser (ANAA) systems produced at the Lawrence Livermore National Laboratory.[226, 227] The MATCI represents a battery-powered thermal cycler with a fluorescent based detection system, which can fit in a "medium-sized" briefcase.[111, 226] A handheld version of ANAA has been used for bacterial detection, producing an audible and visual alert upon a positive result using real-time PCR within 7 minutes.[112] However, both systems still rely on plastic tubes for containment of PCR reagents with volumes between 25 µl and 30 µl therefore the cost saving potential is reduced.

In terms of published research papers, which have been able to successfully integrate the multitude of steps required for genetic analysis, there are relatively few. The work reported by Easley *et al*., demonstrated the a completely integrated genetic analysis device for detection of *Bacillus anthracis* from whole blood samples whereby DNA extraction, PCR and capillary electrophoresis were performed on a single microfluidic device.[192] In addition, Liu *et al*., carried out forensic analysis at a mock crime scene using an integrated microfluidic system capable of producing a CODIS hit within 6 hours of sample collection.[193] While these integrated genetic analysis devices, described in detail in Chapter 1.5, share some similarities with the work presented in this thesis there there are a number of important differences.

The simplistic design of the microfluidic device presented here compared to the more complex four layer glass-PDMS chips reported in the literature offers considerable advantages. The requirement for multiple valves, in both reported systems, increases the complexity of the microfluidic device, making it less amenable to becoming a mass produced device that is single use - an option which would be necessary in the forensic setting due to issues regarding the possibility of contamination from multiuse systems and the need to retain samples as evidence.

Electro-osmotic pumping is used to drive movement of reagents around the microfluidic device eliminating the need for external pumps. In addition, all the necessary reagents, for performing both DNA extraction and PCR, are stored on the microfluidic device. In comparison to the hydrodynamically-driven systems reported in the literature, this methodology should prove more ameanable to the development of a truly portable system. No complex connections between the microfluidic device and the operating system are required, simply the closing of a lid to bring the carbon electrodes within the microfluidic device into contact with the electrical connections on the control system. Storage of the reagents means that the self-contained microfluidic device only requires the DNA sample to be inputted and then device sealed prior to use. This removes the need for

hydrodynamic pumping of solutions onto the device, as and when required, by the current systems.

DNA extraction is not only performed on the microfluidic device, unlike in the Liu *et al.* paper, but uses a silica-based monolith as the solid-phase which has been demonstrated to exhibit greater reproducibility than when silica beads are used. While both contact and non-contact heating methods for thermal cycling have previously been described, the work presented here offers the possibility of using novel microwave heating to provide fast and precise temperature transitions.

In conclusion, the work presented here describes the development of a microfluidic system for performing integrated DNA extraction and amplification. Single use microfluidic devices are described which represent self-contained units that can be pre-loaded with all the necessary reagents for performing DNA extraction and amplification from biological samples. Following manual sample addition, all movement around the device is controlled electrokinetically. A purpose-built control system has been developed which facilitates both electrokinetic movement and thermal cycling using a Peltier heater. While in this present study, the integrated DNA extraction and amplification system has been investigated for forensic analysis, it could easily be adapted for other uses. For example, clinical samples could be analysed for pathogens or disease states by changing the primer sets used and therefore the target nucleic acid sequences.

7 Further Work

In order to improve on the work presented here and to further develop the integrated genetic analysis system, the following further work is proposed:

- There are two main settings in which the proposed integrated genetic analysis system could be used: in a police custody suite or at the scene of a crime. Testing of an initial prototype system would be preferentially carried out on criminal justice samples in a custody suite setting. This would maintain a consistent sample type (buccal swabs) and a more stable environment for initial testing. As a result of this development of an automated interface for direct addition of a buccal swab sample to the microfluidic device is proposed to eliminate the current need for manual injection of the biological sample.

- While the focus of this study has been on the examination of pre-extracted DNA and buccal swab samples there are a wide variety of biological sample types which have the potential to be present at the scene of a crime. DNA extraction and subsequent amplification has been demonstrated on this system from whole and dried blood samples. Further work would focus on DNA extraction from a wider range of biological sample types including urine, fingerprints and semen. The examination of semen samples would require additional treatment through the use of a reducing agent, such as DTT, to break the disulphide bond layer protecting the sperm cell nucleus.

- The work presented here has demonstrated that PCR reagents can be stored on the microfluidic device for at least 8 weeks at 4°C with no reduction in activity. Further work is required to determine the maximum storage time for the PCR reagents in the microfluidic device without loss of activity. A greater potential storage time for the microfluidic device would be advantageous in developing a commercial product as it would provide the customer with greater flexibility.

- The current system involves the amplification of nine forensically relevant loci, present in both standard UK [AmpF*l*STR® SGM Plus®] and US [PowerPlex® 16 System] DNA profiling kits. Further work would be required to increase the number of amplified loci to 16 to anticipate the new European guidelines for DNA profiling, which involves a move away from the current AmpF*l*STR® SGM Plus® DNA profiling system.

- The current integrated genetic analysis system has a Peltier heater incorporated in it to provide the thermal cycling for DNA amplification. Investigation of the use of microwave heating for DNA amplification on a microfluidic device, demonstrated that this non-contact heating method provides significantly faster temperature ramping times. As such further work would aim to replace the Peltier heater in the integrated genetic analysis system with a microwave heating system. In addition it is hoped that the 30 second PCR step temperature hold times currently used in the microwave heating system could be reduced, as the DNA amplification reaction is no longer limited by the thermal transition time, further reducing the overall time for analysis. The current system takes 42 minutes to go through 28 cycles; a reduction to theoretically achievable hold times of 5 seconds at each temperature would mean 28 cycles could be completed in just 7 minutes.

- While integrated DNA extraction and amplification have been demonstrated in a pre-loaded gel-based system using EOP, there is further work required on the optimisation of the integrated process. The DNA profile is no longer as balanced as when DNA amplification is carried out as an isolated technique in an aqueous environment and therefore individual primer concentrations require further adjustment. Also it is possible that there may be some carryover of the ethanol wash solution into the PCR chamber. While it is obviously not sufficient enough to cause inhibition of the DNA amplification reaction it may be responsible for reducing the efficiency of the reaction. This problem could be eliminated by initially directing the TE buffer flow into the waste channel rather than straight into the PCR chamber.

Unfortunately in the current system the electrode configuration on the integrated genetic analysis system prevents this, but modifications could be carried out to enable this to be investigated. This work would ensure that the entire integrated process is as efficient and time-saving as possible whilst generating amplified DNA of sufficient quality to produce a fully interpretable DNA profile.

- Following DNA extraction and amplification, samples are currently analysed using conventional capillary gel electrophoresis techniques. In order to produce a completely integrated genetic analysis system, DNA extraction and amplification on the microfluidic device requires integration with capillary gel electrophoresis and fluorescence detection on the same device. The latter aspects have also been examined as part of the total 'at-scene-of-crime' project, by another PhD student, but lack the required resolution to be able to separate PCR products and therefore require further work to enable the production of a completely integrated system. The aim is to transfer the PCR products by electrokinetic injection into the top of the separation channel prior to separation and detection. Once this complete integration has occurred a compromise can take place between the number of PCR cycles and the sensitivity of the detection system, involving adjusting the number of PCR cycles accordingly, hopefully to smaller number.

- Once the prototype integrated genetic analysis system has been developed further, validation studies would be required if the device was to be used to generate DNA profiles for use in court. This would involve evaluating the system and comparing it against currently used techniques on a large number of samples and ensuring the DNA profile results obtained where equivalent in all cases. In order to eliminate any possibility of contamination between samples, each microfluidic devices would be single use. The device would also be bar-coded so chain of custody could be maintained and in addition some of the DNA would be retained allowing the potential for secondary analysis by the defence if required at a later date.

Publications & Presentations

Papers

Integrated microwave heating with air impingement cooling for rapid PCR amplification using a microfluidic device, **Shaw, K.J.,** Docker, P.T., Yelland, J., Dyer, C.E., Greenman, J., Greenway, G.M., & Haswell, S.J., Lab on a Chip, In press (2009).

Simple practical approach for sample loading prior to DNA extraction using a silica monolith in a microfluidic device, **Shaw, K.J.,** Joyce, D.A., Docker, P.T., Dyer, C.E., Greenman, J., Greenway, G.M., & Haswell, S.J., Lab on a Chip, DOI: 10.1039/B913309G (2009).

The use of carrier RNA to enhance DNA extraction from microfluidic-based silica monoliths, **Shaw, K.J.,** Thain, L., Docker, P.T., Dyer, C.E., Greenman, J., Greenway, G.M., & Haswell, S.J., Analytica Chemica Acta, DOI: 10.1016/j.aca.2009.03.038 (2009).

Development of a bi-functional silica monolith for electro-osmotic pumping and DNA clean-up/extraction using gel-supported reagents in a microfluidic device, Oakley, J.A., **Shaw, K.J.,** Docker, P.T., Dyer, C.E., Greenman, J., Greenway, G.M., & Haswell, S.J., Lab on a Chip, 9: 1596-1600 (2009).

Oral Presentations

Lab-on-a-Chip: DNA profiling, **Shaw, K.J.**, International Crime Science Conference, London (2008).

Poster Presentations

DNA extraction, using carrier RNA, integrated with agarose gel-based PCR in a microfluidic device, **Shaw, K.J.** Oakley, J.A. Docker, P.T. Dyer, C.E. Greenman, J. Greenway, G.M. & Haswell. S.J., Twelfth International Conference on Miniaturised Systems for Chemistry and Life Sciences, San Diego (2008).

Development of a microfluidic device for combined RNA extraction and real-time RT-PCR, Hughes, E.M. **Shaw, K.J.** Docker, P.T. Dyer, C.E. Greenman, J. & Haswell, S.J., Twelfth International Conference on Miniaturised Systems for Chemistry and Life Sciences, San Diego (2008).

Improved efficiency of DNA extraction using carrier RNA, **Shaw, K.J.** Thain, L. Docker, P.T. Dyer, C.E. Greenman, J. Greenway, G.M. & Haswell, S.J., Analytical Research Forum, Hull (2008).

Development of an integrated microfluidic solid-phase DNA extraction and PCR amplification methodology for forensic applications, **Shaw, K.J.** Docker, P.T. Dyer, C.E. Greenman, J. Greenway, G.M. & Haswell, S.J., MicroSystems Biology, Berlin (2007).

On chip DNA extraction and separation for at scene of crime employment, **Shaw, K.J.** Joyce, D.A. Dyer, C.E. & Haswell, S.J., Analytical Research Forum, Glasgow (2007).

References

1. Walker, F. O., Huntington's disease. *Lancet* **2007,** 369, 218-228.

2. Butler, J. M., *Forensic DNA Typing*. Academic Press: 2001.

3. HomeOffice National DNA database. http://www.homeoffice.gov.uk/science-research/using-science/dna-database/ (12th June 2009).

4. National DNA database strategy board. DNA database annual report. **2007**.

5. Watson, J. D.; Crick, F., Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature* **1953,** 171, (4356), 737-738.

6. Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D., *Molecular biology of the cell*. 3rd edition ed.; Garland Publishing Inc: New York & London, **1994**.

7. Belmont, P.; Constant, J.-F.; Demeunynck, M., Nucleic acid conformation diversity: from structure to function & regulation. *Chemical Society Review* **2001,** 30, 70-81.

8. National Blood Service. Frequency of major blood groups in the UK population. http://www.blood.co.uk/pages/all_about.html (27th August 2009).

9. Jeffreys, A. J.; Wilson, V.; Thain, S. L., Hypervariable 'minsatellite' regions in human DNA. *Nature* **1985,** 314, 67-73.

10. Huie, E. M.; Kirshenbaum, M. R.; Trainor, J. L., Oligonucleotides with a nuclease resistent sulfur-based linkage. *Journal of Organic Chemistry* **1992,** 57, (17), 4569-4570.

11. Dale, J. W.; von Schantz, M., *From Genes to Genomes: Concepts and Applications of DNA Technology*. John Wiley & Sons Ltd: **2002.**

12. Applied Biosystems. AmpFlSTR® SGMPlus™ Kit User Manual. **2008**.

13. Tabeling, P., *Introduction to microfluidics*. Oxford University Press: **2005**.

14. Bruus, H., *Theoretical microfluidics*. Oxford University Press: **2008**.

15. Zhang, C. S.; Xu, J. L.; Ma, W. L.; Zheng, W. L., PCR microfluidic devices for DNA amplification. *Biotechnology Advances* **2006,** 24, (3), 243-284.

16. Ong, S.-E.; Zhang, S.; Du, H.; Fu, Y., Fundamental principles and applications of microfluidic systems. *Frontiers in Bioscience* **2008,** 13, 2757-2773.

17. Lee, D. S.; Park, S. H.; Yang, H. S.; Chung, K. H.; Yoon, T. H.; Kim, S. J.; Kim, K.; Kim, Y. T., Bulk-micromachined submicroliter-volume PCR chip with very rapid thermal response and low power consumption. *Lab on a Chip* **2004,** 4, (4), 401-407.

18. Zhang, C. S.; Xing, D., Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. *Nucleic Acids Research* **2007,** 35, (13), 4223-4237.

19. Cho, Y. K.; Lee, J. G.; Park, J. M.; Lee, B. S.; Lee, Y.; Ko, C., One-step pathogen specific DNA extraction from whole blood on a centrifugal microfluidic device. *Lab on a Chip* **2007,** 7, (5), 565-573.

20. Weinberger, R., *Practical capillary electrophoresis*. Academic Press Inc: **1993**.

21. Baker, D. R., *Capillary electrophoresis*. John Wiley & Sons: **1995**.

22. Kang, Y.; Li, D., Electrokinetic motion of particles and cells in microchannels. *Microfluidics & Nanofluidics* **2009,** 6, (4), 431-460.

23. Lee, J. G.; Cheong, K. H.; Huh, N.; Kim, S.; Choi, J. W.; Ko, C., Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification. *Lab on a Chip* **2006,** 6, (7), 886-895.

24. Boom, R.; Sol, C. J. A.; Salimans, M. M. M.; Jansen, C. L.; Wertheimvandillen, P. M. E.; Vandernoordaa, J., Rapid and Simple Method for Purification of Nucleic-Acids. *Journal of Clinical Microbiology* **1990,** 28, (3), 495-503.

25. Chen, X.; Cui, D. F.; Liu, C. C.; Cai, H. Y., Microfluidic biochip for blood cell lysis. *Chinese Journal of Analytical Chemistry* **2006,** 34, (11), 1656-1660.

26. Σεεγer, C.; Batz, H. G.; Orum, H., PNA-mediated purification of PCR amplifiable human genomic DNA from whole blood. *Biotechniques* **1997,** 23, (3), 512-517.

27. Ji, H. M.; Samper, V.; Chen, Y.; Hui, W. C.; Lye, H. J.; Mustafa, F. B.; Lee, A. C.; Cong, L.; Heng, C. K.; Lim, T. M., DNA purification silicon chip. *Sensors and Actuators A-Physical* **2007,** 139, (1-2), 139-144.

28. Wilding, P.; Kricka, L. J.; Cheng, J.; Hvichia, G.; Shoffner, M. A.; Fortina, P., Integrated cell isolation and polymerase chain reaction analysis using silicon microfilter chambers. *Analytical Biochemistry* **1998,** 257, (2), 95-100.

29. Horsman, K. M.; Barker, S. L. R.; Ferrance, J. P.; Forrest, K. A.; Koen, K. A.; Landers, J. P., Separation of sperm and epithelial cells in a microfabricated device: Potential application to forensic analysis of sexual assault evidence. *Analytical Chemistry* **2005,** 77, (3), 742-749.

30. Irimia, D.; Tompkins, R. G.; Toner, M., Single-cell chemical lysis in picoliter-scale closed volumes using a microfabricated device. *Analytical Chemistry* **2004,** 76, (20), 6137-6143.

31. Bienvenue, J. M.; Duncalf, N.; Marchiarullo, D.; Ferrance, J. P.; Landers, J. P., Microchip-based cell lysis and DNA extraction from sperm cells for application to forensic analysis. *Journal of Forensic Sciences* **2006,** 51, (2), 266-273.

32. Heo, J.; Thomas, K. J.; Seong, G. H.; Crooks, R. M., A microfluidic bioreactor based on hydrogel-entrapped E. coli: Cell viability, lysis, and intracellular enzyme reactions. *Analytical Chemistry* **2003,** 75, (1), 22-26.

33. Generelli, S.; Jacquemart, R.; de Rooij, N. F.; Jolicoeur, M.; Koudelka-Hepb, M.; Guenat, O. T., Potentiometric platform for the quantification of cellular potassium efflux. *Lab on a Chip* **2008,** 8, (7), 1210-1215.

34. Khandurina, J.; Jacobson, S. C.; Waters, L. C.; Foote, R. S.; Ramsey, J. M., Microfabricated porous membrane structure for sample concentration and electrophoretic analysis. *Analytical Chemistry* **1999,** 71, (9), 1815-1819.

35. Kim, J.; Jang, S. H.; Jia, G. Y.; Zoval, J. V.; Da Silva, N. A.; Madou, M. J., Cell lysis on a microfluidic CD (compact disc). *Lab on a Chip* **2004,** 4, (5), 516-522.

36. Quinto-Su, P. A.; Lai, H. H.; Yoon, H. H.; Sims, C. E.; Allbritton, N. L.; Venugopalan, V., Examination of laser microbeam cell lysis in a PDMS microfluidic channel using time-resolved imaging. *Lab on a Chip* **2008,** 8, (3), 408-414.

37. Ramadan, Q.; Samper, V.; Poenar, D.; Liang, Z.; Yu, C.; Lim, T. M., Simultaneous cell lysis and bead trapping in a continuous flow microfluidic device. *Sensors and Actuators B-Chemical* **2006,** 113, (2), 944-955.

38. Lu, H.; Schmidt, M. A.; Jensen, K. F., A microfluidic electroporation device for cell lysis. *Lab on a Chip* **2005,** 5, (1), 23-29.

39. Lee, S. W.; Tai, Y. C., A micro cell lysis device. *Sensors and Actuators A-Physical* **1999,** 73, (1-2), 74-79.

40. Khanna, P.; Ramachandran, N.; Yang, J.; Wang, J.; Kumar, A.; Jaroszeski, M.; Bhansali, S., Nanocrystalline diamond microspikes increase the efficiency of ultrasonic cell lysis in a microfluidic device. *Diamond and Related Materials* **2009,** 18, (4), 606-610.

41. Di Carlo, D.; Jeong, K. H.; Lee, L. P., Reagentless mechanical cell lysis by nanscale barbs in microchannels for sample preparation. *Lab on a Chip* **2003,** 3, (4), 287-291.

42. Kim, Y. C.; Kang, J. H.; Park, S. J.; Yoon, E. S.; Park, J. K., Microfluidic biomechanical device for compressive cell stimulation and lysis. *Sensors and Actuators B-Chemical* **2007,** 128, (1), 108-116.

43. Di Carlo, D.; Ionescu-Zanetti, C.; Zhang, Y.; Hung, P.; Lee, L. P., On-chip cell lysis by local hydroxide generation. *Lab on a Chip* **2005,** 5, 171-178.

44. Levison, P. R.; Badger, S. E.; Hathi, P.; Davies, M. J.; Bruce, I. J.; Grimm, V., New approaches to the isolation of DNA by ion-exchange chromatography. *Journal of Chromatography A* **1998,** 827, 337-344.

45. Qiagen, Anion-exchange resins. http://www1.qiagen.com/resources/info/qiagen_purification_technologies_1.aspx (22nd June 2009).

46. Qiagen, QIAamp® DNA Micro Handbook. **2003**.

47. Melzak, K. A.; Sherwood, C. S.; Turner, R. F. B.; Haynes, C. A., Driving forces for DNA adsorption to silica in perchlorate solutions. *Journal of Colloid and Interface Science* **1996,** 181, (2), 635-644.

48. Tian, H. J.; Huhmer, A. F. R.; Landers, J. P., Evaluation of silica resins for direct and efficient extraction of DNA from complex biological matrices in a miniaturized format. *Analytical Biochemistry* **2000,** 283, (2), 175-191.

49. Nawrocki, J., The silanol group and its role in liquid chromatography. *Journal of Chromatography A* **1997,** 779, (1-2), 29-71.

50. Read, S. J., Recovery efficiencies of nucleic acid extraction kits as measured by quantitative LightCyclerTM PCR. *Journal of Clinical Pathology: Molecular Pathology* **2001,** 54, 86-90.

51. Schiffner, L. A.; Bajda, E. J.; Prinz, M.; Sebestyen, J.; Shaler, R.; Caragine, T. A., Optimization of a Simple, Automatable Extraction Method to Recover Sufficient DNA from Low Copy Number DNA Samples for Generation of Short Tandem Repeat Profiles. *Croatian Medical Journal* **2005,** 46, (4), 578-586.

52. Heath, E. M.; Morken, N. W.; Campbell, K. A.; Tkach, D.; Boyd, E. A.; Strom, D. A., Use of Buccal Cells Collected in Mouthwash as a Source of DNA for Clinical Testing. *Archives of Patholology and Laboratory Medicine* **2001,** 125, 127-133.

53. Kishore, R.; Hardy, W. R.; Anderson, V. J.; Sanchez, N. A.; Buoncristiani, M. R., Optimization of DNA extraction from low-yield and degraded samples using the BioRobot® EZ1 and BioRobot® M48. *Journal of Forensic Sciences* **2006,** 51, (5), 1055-1061.

54. Poeckh, T.; Lopez, S.; Overta Fuller, A.; Solomon, M. J.; Larson, R. G., Adsorption and elution characteristics of nucleic acids on silica surfaces and their use in designing a miniaturized purification unit. *Analytical Biochemistry* **2008,** 373, 253-262.

55. Cady, N. C.; Stelick, S.; Batt, C. A., Nucleic acid purification using microfabricated silicon structures. *Biosensors & Bioelectronics* **2003,** 19, (1), 59-66.

56. Hindson, B. J.; Gutierrez, D. M.; Ness, K. D.; Makarewicz, A. J.; Metz, T. R.; Setlur, U. S.; Benett, W. B.; Loge, J. M.; Colston, B. W.; Francis, P. S.; Barnett, N. W.; Dzenitis, J. M., Development of an automated DNA purification module using a microfabricated pillar chip. *Analyst* **2008,** 133, (2), 248-255.

57. West, J.; Boerlin, M.; Jadhav, A. D.; Clancy, E., Silicon microstructure arrays for DNA extraction by solid phase sample contacting at high flow rates. *Sensors and Actuators B-Chemical* **2007,** 126, (2), 664-671.

58. Andersson, H.; van der Wijngaart, W.; Enoksson, P.; Stemme, G., Micromachined flow-through filter-chamber for chemical reactions on beads. *Sensors and Actuators B-Chemical* **2000,** 67, (1-2), 203-208.

59. Sato, K.; Tokeshi, M.; Odake, T.; Kimura, H.; Ooi, T.; Nakao, M.; Kitamori, T., Integration of an immunosorbent assay system: Analysis of secretory human immunoglobulin A on polystyrene beads in a microchip. *Analytical Chemistry* **2000,** 72, (6), 1144-1147.

60. Ceriotti, L.; de Rooij, N. F.; Verpoorte, E., An integrated fritless column for on-chip capillary electrochromatography with conventional stationary phases. *Analytical Chemistry* **2002,** 74, (3), 639-647.

61. Andersson, H.; Jonsson, C.; Moberg, C.; Stemme, G., Self-assembled and self-sorted array of chemically active beads for analytical and biochemical screening. *Talanta* **2002,** 56, (2), 301-308.

62. Malmstadt, N.; Yager, P.; Hoffman, A. S.; Stayton, P. S., A smart microfluidic affinity chromatography matrix composed of poly(N-isopropylacrylamide)-coated beads. *Analytical Chemistry* **2003,** 75, (13), 2943-2949.

63. Lehmann, U.; Vandevyver, C.; Parashar, V. K.; Gijs, M. A. M., Droplet-based DNA purification in a magnetic lab-on-a-chip. *Angewandte Chemie-International Edition* **2006,** 45, (19), 3062-3067.

64. Peyman, S. A.; Iles, A.; Pamme, N., Rapid on-chip multi-step (bio)chemical procedures in continuous flow - manoeuvring particles through co-laminar reagent streams. *Chemical Communications* **2008**, (10), 1220-1222.

65. Zhong, R. T.; Liu, D.; Yu, L. F.; Ye, N.; Dai, Z. P.; Oin, J. H.; Lin, B. C., Fabrication of two-weir structure-based packed columns for on-chip solid-phase extraction of DNA. *Electrophoresis* **2007,** 28, (16), 2920-2926.

66. Geng, T.; Bao, N.; Gall, O. Z.; Lu, C., Modulating DNA adsorption on silica beads using an electrical switch. *Chemical Communications* **2009**, 800-802.

67. Wolfe, K. A.; Breadmore, M. C.; Ferrance, J. P.; Power, M. E.; Conroy, J. F.; Norris, P. M.; Landers, J. P., Toward a microchip-based solid-phase extraction method for isolation of nucleic acids. *Electrophoresis* **2002,** 23, (5), 727-733.

68. Breadmore, M. C.; Wolfe, K. A.; Arcibal, I. G.; Leung, W. K.; Dickson, D.; Giordano, B. C.; Power, M. E.; Ferrance, J. P.; Feldman, S. H.; Norris, P. M.; Landers, J. P., Microchip-based purification of DNA from biological samples. *Analytical Chemistry* **2003,** 75, (8), 1880-1886.

69. Kulinski, M. D.; Mahalanabis, M.; Gillers, S.; Zhang, J. Y.; Singh, S.; Klapperich, C. M., Sample preparation module for bacterial lysis and isolation of DNA from human urine. *Biomedical Microdevices* **2009,** 11, 671-678.

70. Yu, S.; Geng, J.; Zhou, P.; Wang, J.; Feng, A.; Chen, X.; Tong, H.; Hu, J., Application of a new hybrid organic-inorganic monolithic column for efficienct deoxyribonucleic acid purification. *Analytica Chimica Acta* **2008,** 611, 173-181.

71. Chen, X.; Cui, D. F.; Liu, C. C.; Li, H., Fabrication of DNA purification microchip integrated with mesoporous matrix based on MEMS technology. *Microsystem Technologies-Micro-and Nanosystems-Information Storage and Processing Systems* **2008,** 14, (1), 51-57.

72. Ferrance, J. P.; Wu, Q. R.; Giordano, B.; Hernandez, C.; Kwok, Y.; Snow, K.; Thibodeau, S.; Landers, J. P., Developments toward a complete micro-total analysis system for Duchenne muscular dystrophy diagnosis. *Analytica Chimica Acta* **2003,** 500, (1-2), 223-236.

73. Wu, Q. R.; Bienvenue, J. M.; Hassan, B. J.; Kwok, Y. C.; Giordano, B. C.; Norris, P. M.; Landers, J. P.; Ferrance, J. P., Microchip-based macroporous silica sol-gel monolith for efficient isolation of DNA from clinical samples. *Analytical Chemistry* **2006,** 78, (16), 5704-5710.

74. Kato, M.; Sakai-Kato, K.; Toyo'oka, T.; Dulay, M. T.; Quirino, J. P.; Bennett, B. D.; Zare, R. N., Effect of preparatory conditions on the performance of photopolymerized sol-gel monoliths for capillary electrochromatography. *Journal of Chromatography A* **2002,** 961, (1), 45-51.

75. Wen, J.; Guillo, C.; Ferrance, J. P.; Landers, J. P., DNA extraction using a tetramethyl orthosilicate-grafted photopolymerized monolithic solid phase. *Analytical Chemistry* **2006,** 78, (5), 1673-1681.

76. Wen, J.; Guillo, C.; Ferrance, J. P.; Landers, J. P., Microfluidic chip-based protein capture from human whole blood using octadecyl (C18) silica beads for nucleic acid analysis from large volume samples. *Journal of Chromatography A* **2007,** 1171, (1-2), 29-36.

77. Wen, J.; Guillo, C.; Ferrance, J. P.; Landers, J. P., Microfluidic-based DNA purification in a two-stage, dual-phase microchip containing a reversed-phase and a photopolymerized monolith. *Analytical Chemistry* **2007,** 79, (16), 6135-6142.

78. Chen, X.; Cui, D. F.; Liu, C. C., On-line cell lysis and DNA extraction on a microfluidic biochip fabricated by microelectromechanical system technology. *Electrophoresis* **2008,** 29, 1844-1851.

79. Nakagawa, T.; Tanaka, T.; Niwa, D.; Osaka, T.; Takeyama, H.; Matsunaga, T., Fabrication of amino silane-coated microchip for DNA extraction from whole blood. *Journal of Biotechnology* **2005,** 116, (2), 105-111.

80. Cao, W. D.; Easley, C. J.; Ferrance, J. P.; Landers, J. P., Chitosan as a polymer for pH-induced DNA capture in a totally aqueous system. *Analytical Chemistry* **2006,** 78, (20), 7222-7228.

81. Lien, K. Y.; Liu, C. J.; Lin, Y. C.; Kuo, P. L.; Lee, G. B., Extraction of genomic DNA and detection of single nucleotide polymorphism genotyping utilizing an integrated magnetic bead-based microfluidic platform. *Microfluidics & Nanofluidics* **2009,** 6, (4), 539-555.

82. Witek, M. A.; Llopis, S. D.; Wheatley, A.; McCarley, R. L.; Soper, S. A., Purification and preconcentration of genomic DNA from whole cell lysates using photoactivated polycarbonate (PPC) microfluidic chips. *Nucleic Acids Research* **2006,** 34, (10).

83. Kim, J.; Gale, B. K., Quantitative and qualitative analysis of a microfluidic DNA extraction system using a nanoporous AlOx membrane *Lab on a Chip* **2008,** 8, (9), 1516-1523.

84. Yeung, S. W.; Hsing, I. M., Manipulation and extraction of genomic DNA from cell lysate by functionalized magnetic particles for lab on a chip applications. *Biosensors & Bioelectronics* **2006,** 21, (7), 989-997.

85. Archer, M. J.; Lin, B. C.; Wang, Z.; Stenger, D. A., Magnetic bead-based solid phase for selective extraction of genomic DNA. *Analytical Biochemistry* **2006,** 355, (2), 285-297.

86. Prinz, C.; Tegenfeldt, J. O.; Austin, R. H.; Cox, E. C.; Sturm, J. C., Bacterial chromosome extraction and isolation. *Lab on a Chip* **2002,** 2, 207-212.

87. Park, S. R.; Swerdlow, H., Concentration of DNA in a flowing stream for high-sensitivity capillary electrophoresis. *Analytical Chemistry* **2003,** 75, (17), 4467-4474.

88. Burtis, C. A.; Ashwood, E. R.; Bruns, D. E., *Fundamentals of Clinical Chemistry*. 6th ed.; Saunders Elsevier: **2008**.

89. Applied Biosystems, TaqMan probes. {http://www3.appliedbiosystems.com/AB_Home/applicationstechnologies/Real-TimePCR/TaqManvsSYBRGreenChemistries/index.htm} (16th June 2009).

90. Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T., Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* **2000,** 28, (12), e63i-vii.

91. Polymerase chain reaction. http://oceanexplorer.noaa.gov/explorations/04etta/background/dna/media/dna_1.html (27th July 2009).

92. McCusker, J.; Dawson, M. T.; Noone, D.; Gannon, F.; Smith, T., Improved Method for Direct PCR Amplification from Whole-Blood. *Nucleic Acids Research* **1992,** 20, (24), 6747-6747.

93. Huang, H.; Bu, Y.; Zhou, G. H., Single-tube-genotyping of gastric cancer related SNPs by directly using whole blood and paper-dried blood as starting materials. *World Journal of Gastroenterology* **2006,** 12, (24), 3814-3820.

94. Zhang, N. Y.; Tan, H. D.; Yeung, E. S., Automated and integrated system for high-throughput DNA genotyping directly from blood. *Analytical Chemistry* **1999,** 71, (6), 1138-1145.

95. Auroux, P. A.; Koc, Y.; deMello, A.; Manz, A.; Day, P. J. R., Miniaturised nucleic acid analysis. *Lab on a Chip* **2004,** 4, (6), 534-546.

96. Krishnan, M.; Ugaz, V. M.; Burns, M. A., PCR in a Rayleigh-Benard Convection Cell. *Science* **2002,** 298, 793.

97. Oda, R. P.; Strausbauch, M. A.; Huhmer, A. F. R.; Borson, N.; Jurrens, S. R.; Craighead, J.; Wettstein, P. J.; Eckloff, B.; Kline, B.; Landers, J. P., Infrared-mediated thermocycling for ultrafast polymerase chain reaction amplification of DNA. *Analytical Chemistry* **1998,** 70, (20), 4361-4368.

98. Easley, C. J.; Humphrey, J. A. C.; Landers, J. P., Thermal isolation of microchip reaction chambers for rapid non-contact DNA amplification. *Journal of Micromechanics & Microengineering* **2007,** 17, (9), 1758-1766.

99. Roper, M. G.; Easley, C. J.; Legendre, L. A.; Humphrey, J. A. C.; Landers, J. P., Infrared Temperature Control System for a Completely Noncontact Polymerase Chain Reaction in Microfluidic Chips. *Analytical Chemistry* **2007,** 79, 1294-1300.

100. Ohashi, T.; Kuyama, H.; Hanafusa, N.; Togawa, Y., A simple device using magnetic transportation for droplet-based PCR. *Biomedical Microdevices* **2007,** 9, (5), 695-702.

101. Neuzil, P.; Zhang, C.; Pipper, J.; Oh, S.; Zhuo, L., Ultra fast miniaturized real-time PCR: 40 cycles in less than six minutes. *Nucleic Acids Research* **2006,** 34, (11), e77.

102. Shin, Y. S.; Cho, K.; Lim, S. H.; Chung, S.; Park, S. J.; Chung, C.; Han, D. C.; Chang, J. K., PDMS-based micro PCR chip with parylene coating. *Journal of Micromechanics & Microengineering* **2003,** 13, (5), 768-774.

103. Xiang, Q.; Xu, B.; Fu, R.; Li, D., Real time PCR on disposable PDMS chip with a miniaturized thermal cycler. *Biomedical Microdevices* **2005,** 7, (4), 273-279.

104. Prakash, A. R.; Adamiab, S.; Sieben, V.; Pilarski, P.; Pilarski, L. M.; Backhouse, C. J., Small volume PCR in PDMS biochips with integrated fluid control and vapour barrier. *Sensors and Actuators B-Chemical* **2006,** 113, 398-409.

105. Giordano, B. C.; Ferrance, J.; Swedberg, S.; Huhmer, A. F. R.; Landers, J. P., Polymerase chain reaction in polymeric microchips: DNA amplification in less than 240 seconds. *Analytical Biochemistry* **2001,** 291, (1), 124-132.

106. Wheeler, E. K.; Benett, W.; Stratton, P.; Richards, J.; Chen, A.; Christian, A.; Ness, K. D.; Ortega, J.; Li, L. G.; Weisgraber, T. H.; Goodson, K.; Milanovich, F., Convectively driven polymerase chain reaction thermal cycler. *Analytical Chemistry* **2004,** 76, (14), 4011-4016.

107. Kalinina, O.; Lebedeva, I.; Brown, J.; Silver, J., Nanoliter scale PCR with TaqMan detection. *Nucleic Acids Research* **1997,** 25, (10), 1999-2004.

108. Ke, C.; Berney, H.; Mathewson, A.; Sheehan, M. M., Rapid amplification for the detection of Mycobacterium tuberculosis using a non-contact heating method in a silicon microreactor based thermal cycler *Sensors and Actuators B-Chemical* **2004,** 102, (2), 308-314.

109. Braun, D.; Goddard, N. L.; Libchaber, A., Exponential DNA Replication by Laminar Convection. *Physical Review Letters* **2003,** 91, (15), 158103-1-158103-4.

110. Northrup, M. A.; Benett, B.; Hadley, D.; Landre, P.; Lehew, S.; Richards, J.; Stratton, P., A miniature analytical instrument for nucleic acids based on micromachined silicon reaction chambers. *Analytical Chemistry* **1998,** 70, (5), 918-922.

111. Belgrader, P.; Smith, J. K.; Weedn, V. W.; Northrup, M. A., Rapid PCR for Identity Testing Using a Battery-Powered Miniature Thermal Cycler. *Journal of Forensic Sciences* **1998,** 43, (2), 315-319.

112. Belgrader, P.; Benett, W.; Hadley, D.; Richards, J.; Stratton, P.; Mariella, R.; Milanovich, F., PCR Detection of Bacteria in Seven Minutes. *Science* **1999,** 284, 449-450.

113. Belgrader, P.; Young, S.; Yuan, B.; Primeau, M.; Christel, L. A.; Pourahmadi, F.; Northrup, M. A., A battery-powered notebook thermal cycler for rapid multiplex real time PCR analysis. *Analytical Chemistry* **2001,** 73, (2), 286-289.

114. Higgins, J. A.; Nasarabadi, S.; Karns, J. S.; Shelton, D. R.; Cooper, M.; Gbakima, A.; Koopman, R. P., A handheld real time thermal cycler for bacterial pathogen detection. *Biosensors & Bioelectronics* **2003,** 18, (9), 1115-1123.

115. Daniel, J. H.; Iqbal, S.; Millington, R. B.; Moore, D. F.; Lowe, C. R.; Leslie, D. L.; Lee, M. A.; Pearce, M. J., Silicon microchambers for DNA amplification. *Sensors and Actuators A-Physical* **1998,** 71, (1-2), 81-88.

116. Lee, T. M. H.; Hsing, I.-M.; Lao, A. I. K.; Carles, M. C., A Miniaturized DNA Amplifier: Its Application in Traditional Chinese Medicine. *Analytical Chemistry* **2000,** 72, 4242-4247.

117. Zhao, Z.; Cui, Z.; Cui, D. F.; Xia, S. H., Monolithically integrated PCR biochip for DNA amplification. *Sensors and Actuators A-Physical* **2003,** 108, (1-3), 162-167.

118. Felbel, J.; Bieber, I.; Pipper, J.; Kohler, J. M., Investigations on the compatibility of chemically oxidized silicon (SiOx)-surfaces for applications towards chip-based polymerase chain reaction. *Chemical Engineering Journal* **2004,** 101, (1-3), 333-338.

119. Wilding, P.; Shoffner, M. A.; Kricka, L. J., PCR in a Silicon Microstructure. *Clinical Chemistry* **1994,** 40, (9), 1815-1818.

120. Shoffner, M. A.; Cheng, J.; Hvichia, G. E.; Kricka, L. J.; Wilding, P., Chip PCR .1. Surface passivation of microfabricated silicon-glass chips for PCR. *Nucleic Acids Research* **1996,** 24, (2), 375-379.

121. Yoon, D. S.; Lee, Y. S.; Lee, Y.; Cho, H. J.; Sung, S. W.; Oh, K. W.; Cha, J.; Lim, G., Precise temperature control and rapid thermal cycling in a micromachined DNA polymerase chain reaction chip. *Journal of Micromechanics & Microengineering* **2002,** 12, (6), 813-823.

122. Erill, I.; Campoy, S.; Erill, N.; Barbe, J.; Aguilo, J., Biochemical analysis and optimization of inhibition and adsorption phenomena in glass-silicon PCR-chips. *Sensors and Actuators B-Chemical* **2003,** 96, (3), 685-692.

123. Lin, Y. C.; Yang, C. C.; Huang, M. Y., Simulation and experimental validation of micro polymerase chain reaction chips. *Sensors and Actuators B-Chemical* **2000,** 71, (1-2), 127-133.

124. El-Ali, J.; Perch-Nielsen, I. R.; Poulsen, C. R.; Bang, D. D.; Telleman, P.; Wolff, A., Simulation and experimental validation of a SU-8 based PCR thermocycler chip with integrated heaters and temperature sensor. *Sensors and Actuators A-Physical* **2004,** 110, (1-3), 3-10.

125. Bruckner-Lea, C. J.; Tsukuda, T.; Dockendorff, B.; Follansbee, J. C.; Kingsley, M. T.; Ocampo, C.; Stults, J. R.; Chandler, D. P., Renewable microcolumns for automated DNA purification and flow-through amplification: from sediment samples through polymerase chain reaction. *Analytica Chimica Acta* **2002,** 469, (1), 129-140.

126. Belgrader, P.; Elkin, C. J.; Brown, S. B.; Nasarabadi, S. N.; Langlois, R. G.; Milanovich, F. P.; Colston, B. W.; Marshall, G. D., A reusable flow-through polymerase chain reaction instrument for the continuous monitoring of infectious biological agents. *Analytical Chemistry* **2003,** 75, (14), 3446-3450.

127. Gong, H.; Ramalingam, N.; Chen, L.; Che, J.; Wang, Q.; Wang, Y.; Yang, X.; Yap, P. H. E.; Neo, C. H., Microfluidic handling of PCR solution and DNA amplification on a reaction chamber array biochip. *Biomedical Microdevices* **2006,** 8, 167-176.

128. Yu, X. M.; Zhang, D. C.; Li, T.; Hao, L.; Li, X. H., 3-D microarrays biochip for DNA amplification in polydimethylsiloxane (PDMS) elastomer. *Sensors and Actuators A-Physical* **2003,** 108, (1-3), 103-107.

129. Wu, Z.-Y.; Chen, K.; Qu, B.-Y.; Tian, X.-X.; Wang, X.-J.; Fang, F., A thermostat chip of indium tin oxide glass substrate for static polymerase chain reaction and in situ real time fluorescence monitoring. *Analytica Chimica Acta* **2008,** 610, 89-96.

130. Marcus, J. S.; Anderson, W. F.; Quake, S. R., Parallel picoliter RT-PCR assays using microfluidics. *Analytical Chemistry* **2006,** 78, (3), 956-958.

131. Liao, C. S.; Lee, G. B.; Wu, J. J.; Chang, C. C.; Hsieh, T. M.; Huang, F. C.; Luo, C. H., Micromachined polymerase chain reaction system for multiple DNA amplification of upper respiratory tract infectious diseases. *Biosensors & Bioelectronics* **2005,** 20, (7), 1341-1348.

132. Xiang, Q.; Xu, B.; Li, D., Miniature real time PCR on chip with multi-channel fiber optical fluorescence detection module. *Biomedical Microdevices* **2007,** 9, (4), 443-449.

133. Taylor, T. B.; WinnDeen, E. S.; Picozza, E.; Woudenberg, T. M.; Albin, M., Optimization of the performance of the polymerase chain reaction in silicon-based microstructures. *Nucleic Acids Research* **1997,** 25, (15), 3164-3168.

134. Taylor, T. B.; Harvey, S. E.; Albin, M.; Lebak, L.; Ning, Y.; Mowat, I.; Schuerlien, T.; Principe, E., Process Control for Optimal PCR Performance in Glass Microstructures. *Biomedical Microdevices* **1998**.

135. Nagai, H.; Murakami, Y.; Morita, Y.; Yokoyama, K.; Tamiya, E., Development of a microchamber array for picoliter PCR. *Analytical Chemistry* **2001,** 73, (5), 1043-1047.

136. Nagai, H.; Murakami, Y.; Yokoyama, K.; Tamiya, E., High-throughput PCR in silicon based microchamber array. *Biosensors & Bioelectronics* **2001,** 16, (9-12), 1015-1019.

137. Zou, Q. B.; Miao, Y. B.; Chen, Y.; Sridhar, U.; Chong, C. S.; Chai, T. C.; Tie, Y.; Teh, C. H. L.; Lim, T. M.; Heng, C., Micro-assembled multi-chamber thermal cycler for low-cost reaction chip thermal multiplexing. *Sensors and Actuators A-Physical* **2002,** 102, (1-2), 114-121.

138. Consolandi, C.; Severgnini, M.; Frosini, A.; Caramenti, G.; De Fazio, M.; Ferrara, F.; Zocco, A.; Fischetti, A.; Palmieri, M.; De Bellis, G., Polymerase chain reaction of 2-kb cyanobacterial gene and human anti-1-chymotrypsin gene from genomic DNA on the In-Check single-use microfabricated silicon chip. *Analytical Biochemistry* **2006,** 353, 191-197.

139. Zou, Z.-Q.; Chen, X.; Jin, Q.-H.; Yang, M.-S.; Zhao, J.-L., A novel miniaturized PCR multi-reactor array fabricated using flip-chip bonding techniques. *Journal of Micromechanics and Microengineering* **2005,** 15, 1476-1481.

140. Dettloff, R.; Yang, E.; Rulison, A.; Chow, A., Nucleic acid amplification of individual molecules in a microfluidic device. *Analytical Chemistry* **2008,** 80, 4208-4213.

141. Beer, N. R.; Hindson, B. J.; Wheeler, E. K.; Hall, S. B.; Rose, K. A.; Kennedy, I. M.; Colston, B. W., On-chip, real-time, single-copy polymerase chain reaction in picoliter droplets. *Analytical Chemistry* **2007,** 79, (22), 8471-8475.

142. Hashimoto, M.; Chen, P. C.; Mitchell, M. W.; Nikitopoulos, D. E.; Soper, S. A.; Murphy, M. C., Rapid PCR in a continuous flow device. *Lab on a Chip* **2004,** 4, (6), 638-645.

143. Chen, P.-C.; Nikitopoulos, D. E.; Soper, S. A.; Murphy, M. C., Temperature distribution effects on micro-CFPCR performance. *Biomedical Microdevices* **2008,** 10, 141-152.

144. Chou, C. F.; Changrani, R.; Roberts, P.; Sadler, D.; Burdon, J.; Zenhausern, F.; Lin, S.; Mulholland, A.; Swami, N.; Terbrueggen, R., A miniaturized cyclic PCR device - modeling and experiments. *Microelectronic Engineering* **2002,** 61-2, 921-925.

145. Kopp, M. U.; DeMello, A.; Manz, A., Chemical Amplification: Continuous-Flow PCR on a Chip. *Science* **1998,** 280, 1046-1048.

146. Obeid, P. J.; Christopoulos, T. K.; Crabtree, H. J.; Backhouse, C. J., Microfabricated device for DNA and RNA amplification by continuous-flow polymerase chain reaction and reverse transcription-polymerase chain reaction with cycle number selection. *Analytical Chemistry* **2003,** 75, (2), 288-295.

147. Crews, N.; Wittwer, C.; Gale, B., Continuous-flow thermal gradient PCR. *Biomedical Microdevices* **2008,** 10, (2), 187-195.

148. Crews, N.; Wittwer, C.; Palais, R.; Gale, B., Product differentiation during continuous-flow thermal gradient PCR. *Lab on a Chip* **2008,** 8, 919-924.

149. Fukuba, T.; Yamamoto, T.; Naganuma, T.; Fujii, T., Microfabricated flow-through device for DNA amplification - towards in situ gene analysis. *Chemical Engineering Journal* **2004,** 101, (1-3), 151-156.

150. Yu, C.; Liang, W.; Kuan, I.; Wei, C.; Gu, W., Fabrication and characterization of a flow-through PCR device with integrated chromium resistive heaters. *Journal of the Chinese Institute of Chemical Engineers* **2007,** 38, 333-339.

151. Kuan, I.; Gua, W.; Wu, J.; Wei, C.; Chenc, K.; Yua, C., Effects of grafting poly(ethylene oxide) on the amplification efficiency of a poly(dimethylsiloxane)-based flow-through PCR device. *Chemical Engineering Journal* **2008,** 143, 326-330.

152. Kiss, M. M.; Ortoleva-Donnelly, L.; Beer, N. R.; Warner, J.; Bailey, C. G.; Colston, B. W.; Rothberg, J. M.; Link, D. R.; Leamon, J. H., High-throughput quantitative polymerase chain reaction in picoliter droplets. *Analytical Chemistry* **2008,** 80, 8975-8981.

153. Yang, J. N.; Liu, Y. J.; Rauch, C. B.; Stevens, R. L.; Liu, R. H.; Lenigk, R.; Grodzinski, P., High sensitivity PCR assay in plastic micro reactors. *Lab on a Chip* **2002,** 2, (4), 179-187.

154. Sun, K.; Yamaguchi, A.; Ishida, Y.; Matsuo, S.; Misawa, H., A heater-integrated transparent microchannel chip for continuous-flow PCR. *Sensors and Actuators B-Chemical* **2002,** 84, (2-3), 283-289.

155. Schneegass, I.; Brautigam, R.; Kohler, J. M., Miniaturized flow-through PCR with different template types in a silicon chip thermocycler. *Lab on a Chip* **2001,** 1, (1), 42-49.

156. Tsai, N.-C.; Sue, C.-Y., SU-8 based continuous-flow RT-PCR bio-chips under high-precision temperature control. *Biosensors & Bioelectronics* **2006,** 22, 313-317.

157. Dorfman, K. D.; Chabert, M.; Codarbox, J. H.; Rousseau, G.; De Cremoux, P.; Viovy, J.-L., Contamination-free continuous microfluidic polymerase chain reaction for quantitative and clinical applications. *Analytical Chemistry* **2005,** 77, 3700-3704.

158. Park, N.; Kim, S.; Hahn, J. H., Cylindrical compact thermal-cycling device for continuous-flow polymerase chain reaction. *Analytical Chemistry* **2003,** 75, (21), 6029-6033.

159. Gonzalez, A.; Ciobanu, D.; Sayers, M.; Sirr, N.; Dalton, T.; Davies, M., Gene transcript amplification from cell lysates in continuous-flow microfluidic devices. *Biomedical Microdevices* **2007,** 9, (5), 729-736.

160. Nakano, H.; Matsuda, K.; Yohda, M.; Nagamune, T.; Endo, I.; Yamane, T., High speed polymerase chain reaction in constant flow. *Bioscience, Biotechnology & Biochemistry* **1994,** 58, (2), 349-352.

161. Curcio, M.; Roeraade, J., Continuous segmented-flow polymerase chain reaction for high-throughput miniaturized DNA amplification. *Analytical Chemistry* **2003,** 75, (1), 1-7.

162. Wang, C.-H.; Chen, Y.-Y.; Liao, C.-S.; Hsieh, T.-M.; Luo, C.-H.; Wu, J.-J.; Lee, H.-H.; Lee, G.-B., Circulating polymerase chain reaction chips utilizing multiple-membrane activation. *Journal of Micromechanics and Microengineering* **2007,** 17, 367-675.

163. Liu, J.; Enzelberger, M.; Quake, S., A nanoliter rotary device for polymerase chain reaction. *Electrophoresis* **2002,** 23, (10), 1531-1536.

164. Sun, Y.; Kwok, Y. C.; Nguyen, N. T., A circular ferrofluid driven microchip for rapid polymerase chain reaction. *Lab on a Chip* **2007,** 7, (8), 1012-1017.

165. Chen, J.; Wabuyele, M.; Chen, H.; Patterson, D.; Hupert, M.; Shadpour, H.; Nikitopoulos, D.; Soper, S. A., Electrokinetically synchronized polymerase chain reaction microchip fabricated in polycarbonate. *Analytical Chemistry* **2005,** 77, 658-666.

166. Chen, Z.; Qian, S.; Abrams, W. R.; Malamud, D.; Bau, H. H., Thermosiphon-based PCR reactor: experiment and modeling. *Analytical Chemistry* **2004,** 76, 3707-3715.

167. Chen, L.; West, J.; Auroux, P. A.; Manz, A.; Day, P. J. R., Ultrasensitive PCR and real-time detection from human genomic samples using a bidirectional flow microreactor. *Analytical Chemistry* **2007,** 79, (23), 9185-9190.

168. Frey, O.; Bonneick, S.; Hierlemann, A.; Lichtenberg, J., Autonomous microfluidic multi-channel chip for real-time PCR with integrated liquid handling. *Biomedical Microdevices* **2007,** 9, (5), 711-718.

169. Hu, G. Q.; Xiang, Q.; Fu, R.; Xu, B.; Venditti, R.; Li, D. Q., Electrokinetically controlled real-time polymerase chain reaction in microchannel using Joule heating effect. *Analytica Chimic Acta* **2006,** 557, (1-2), 146-151.

170. Chiou, J.; Matsudaira, P.; Sonin, A.; Ehrlich, D., A closed cycle capillary polymerase chain reaction machine. *Analytical Chemistry* **2001,** 73, (9), 2018-2021.

171. Zhang, Y.; Ozdemira, P., Microfluidic DNA amplification—A review. *Analytica Chimica Acta* **2009,** 638, (2), 115-125.

172. Schaerli, Y.; Wootton, R. C.; Robinson, T.; Stein, V.; Dunsby, C.; Neil, M. A. A.; French, P. M. W.; deMello, A. J.; Abell, C.; Hollfelder, F., Continuous-Flow Polymerase Chain Reaction of Single-Copy DNA in Microfluidic Microdroplets *Analytical Chemistry* **2009,** 81, 302-306.

173. Liu, J.; Hansen, C.; Quake, S. R., Solving the "world-to-chip" interface problem with a microfluidic matrix. *Analytical Chemistry* **2003,** 75, (18), 4718-4723.

174. Orrling, K.; Nilsson, P.; Gullbergh, M.; Larhed, M., An efficient method to perform milliliter-scale PCR utilizing highly controlled microwave thermocycling. *Chemical Communications* **2004**, (7), 790-791.

175. Prakash, A. R.; Amrein, M.; Kaler, K., Characteristics and impact of Taq enzyme adsorption on surfaces in microfluidic devices. *Microfluidics & Nanofluidics* **2008,** 4, (4), 295-305.

176. Wilding, P.; Shoffner, M. A.; Cheng, J.; Hvichia, G.; Kricka, L. J., Thermal Cycling and Surface Passivation of Micromachined Devices for PCR. *Clinical Chemistry* **1995,** 41, (9), 1367-1368.

177. Giordano, B. C.; Copeland, E. R.; Landers, J. P., Towards dynamic coating of glass microchip chambers for amplifying DNA via the polymerase chain reaction. *Electrophoresis* **2001,** 22, (2), 334-340.

178. Chiari, M.; Cretich, M.; Horvath, J., A new absorbed coating for DNA fragment analysis by capillary electrophoresis. *Electrophoresis* **2000,** 21, (8), 1521-1526.

179. Prakash, R.; Kaler, K., An integrated genetic analysis microfluidic platform with valves and a PCR chip reusability method to avoid contamination. *Microfluidics & Nanofluidics* **2007,** 3, (2), 177-187.

180. Lagally, E. T.; Scherer, J. R.; Blazej, R. G.; Toriello, N. M.; Diep, B. A.; Ramchandani, M.; Sensabaugh, G. F.; Riley, L. W.; Mathies, R. A., Integrated portable genetic analysis microsystem for pathogen/infectious disease detection. *Analytical Chemistry* **2004,** 76, (11), 3162-3170.

181. Kricka, L. J.; Wilding, P., Microchip PCR. *Analytical and Bioanalytical Chemistry* **2003,** 377, (5), 820-825.

182. Kuan, I.; Gua, W.; Wu, J.; Wei, C.; Chenc, K.; Yua, C., Effects of grafting poly(ethylene oxide) on the amplification efficiency of a poly(dimethylsiloxane)-based flow-through PCR device. *Chemical Engineering Journal* **2008,** 143, 326-330.

183. Hong, J. W.; Fujii, T.; Seki, M.; Yamamoto, T.; Endo, I., Integration of gene amplification and capillary gel electrophoresis on a polydimethylsiloxane-glass hybrid microchip. *Electrophoresis* **2001,** 22, (2), 328-333.

184. Invitrogen, *qPCR Handbook*; **2009**.

185. Northrup, M. A.; Ching, M. T.; White, R. M.; Watson, R. T., DNA amplification in a microfabricated reaction chamber. *Transducer* **1993,** Seventh international conference on solid state sensors and actuators, 924-926.

186. Woolley, A. T.; Hadley, D.; Landre, P.; deMello, A. J.; Mathies, R. A.; Northrup, M. A., Functional integration of PCR amplification and capillary electrophoresis in a microfabricated DNA analysis device. *Analytical Chemistry* **1996,** 68, (23), 4081-4086.

187. Yuen, P. K.; Kricka, L. J.; Fortina, P.; Panaro, N. J.; Sakazume, T.; Wilding, P., Microchip module for blood sample preparation and nucleic acid amplification reactions. *Genome Research* **2001,** 11, (3), 405-412.

188. Cheong, K. H.; Yi, D. K.; Lee, J. G.; Park, J. M.; Kim, M. J.; Edel, J. B.; Ko, C., Gold nanoparticles for one step DNA extraction and real-time PCR of pathogens in a single chamber. *Lab on a Chip* **2008,** 8, (5), 810-813.

189. Liu, R. H.; Yang, J. N.; Lenigk, R.; Bonanno, J.; Grodzinski, P., Self-contained, fully integrated biochip for sample preparation, polymerase chain reaction amplification, and DNA microarray detection. *Analytical Chemistry* **2004,** 76, (7), 1824-1831.

190. Cady, N. C.; Stelick, S.; Kunnavakkam, M. V.; Batt, C. A., Real-time PCR detection of Listeria monocytogenes using an integrated microfluidics platform. *Sensors and Actuators B-Chemical* **2005,** 107, (1), 332-341.

191. Legendre, L. A.; Bienvenue, J. M.; Roper, M. G.; Ferrance, J. P.; Landers, J. P., A simple, valveless microfluidic sample preparation device for extraction and amplification of DNA from nanoliter-volume samples. *Analytical Chemistry* **2006,** 78, (5), 1444-1451.

192. Easley, C. J.; Karlinsey, J. M.; Bienvenue, J. M.; Legendre, L. A.; Roper, M. G.; Feldman, S. H.; Hughes, M. A.; Hewlett, E. L.; Merkel, T. J.; Ferrance, J.; Landers, J. P., A fully integrated microfluidic genetic analysis system with sample-in-answer-out capability. *Proceedings of the National Academy of Sciences of the United States of America* **2006,** 103, (51), 19272-19277.

193. Liu, P.; Yeung, S. H. I.; Crenshaw, K. A.; Crouse, C. A.; Scherer, J. R.; Mathies, R. A., Real-time forensic DNA analysis at a crime scene using a portable microchip analyzer. *Forensic Science International: Genetics* **2008,** 2, 301–309.

194. McCreedy, T., Fabrication techniques and materials commonly used for the production of microreactors and micro total analytical systems. *Trac-Trends in Analytical Chemistry* **2000,** 19, (6), 396-401.

195. Ahn, S. J.; Costa, J.; Emanuel, J. R., PicoGreen quantitation of DNA: Effective evaluation of samples pre- or post-PCR. *Nucleic Acids Research* **1996,** 24, (13), 2623-2625.

196. Krenke, B. E.; Tereba, A.; Anderson, S. J.; Buel, E.; Culhane, S.; Finis, C. J.; Tomsey, C. S.; Zachetti, J. M.; Masibay, A.; Rabbach, D. R.; Amiott, E. A.; Sprecher, C. J., Validation of a 16-locus fluorescent multiplex system. *Journal of Forensic Sciences* **2002,** 47, (4), 773-785.

197. Drebushchak, V. A., The peltier effect. *Journal of Thermal Analysis & Colorimetry* **2008,** 91, (1), 311-315.

198. Iofee, A. F., *Semiconductor thermoelements and thermoelectric cooling*. Infosearch Limited: London, **1956**.

199. IXBTLabs Peltier coolers. http://ixbtlabs.com/articles/peltiercoolers/p3.gif (24th June 2009).

200. Martignoni, M.; Groothuis, G.; de Kanter, R., Comparison of mouse and rat cytochrome P450-mediated metabolism in liver and intestine. *Drug Metabolism and Disposition* **2006,** 34, 1047-1054.

201. Dulay, M. T.; Quirino, J. P.; Bennett, B. D.; Kato, M.; Zare, R. N., Photopolymerized sol-gel monoliths for capillary electrochromatography. *Analytical Chemistry* **2001,** 73, (16), 3921-3926.

202. Christensen, P. D.; Johnson, S. W. P.; McCreedy, T.; Skelton, V.; Wilson, N. G., The fabrication of micro-porous silica structures for micro-reactor technology. *Analytical Communications* **1998,** 35, (10), 341-343.

203. Poole, C. F.; Poole, S. K., *Chromatography today*. 1 ed.; Elsevier Science Publishers B.V.: **1991**.

204. Claveau, S.; Sasseville, M.; Beauregard, M., Alcohol-mediated error-prone PCR. *DNA and Cell Biology* **2004,** 23, (22), 789.

205. Robertson, R. M.; Laib, S.; Smith, D. E., Diffusion of isolated DNA molecules: dependance on length and topology. *Proceedings of the National Academy of Sciences of the United States of America* **2006,** 103, (19), 7310-7314.

206. Niu, X. Z.; Zhang, B.; Marszalek, R. T.; Ces, O.; Edel, J. B.; Kluga, D. R.; deMello, A. J., Droplet-based compartmentalization of chemically separated components in two-dimensional separations. *Chemmical Communications* **2009,** 41, 6159-6161.

207. Pamme, N. Single particle analysis in microfluidic chips. Thesis, Imperial College London, London, **2003**.

208. Hartshorne, H.; Backhouse, C. J.; Lee, W. E., Ferrofluid-based microchip pump and valve. *Sensors and Actuators B-Chemical* **2004,** 99, 592-600.

209. Yeung, S. H. I.; Greenspoon, S. A.; McGuckian, A.; Crouse, C. A.; Emrich, C. A.; Ban, J.; Mathies, R. A., Rapid and High-Throughput Forensic Short Tandem Repeat Typing Using a 96-Lane Microfabricated Capillary Array Electrophoresis Microdevice. *Journal of Forensic Sciences* **2006,** 51, (4), 740-747.

210. Baldwin, S. J.; Bramhall, J. L.; Ashby, C. A.; Yue, L.; Murdock, P. R.; Hood, S. R.; Ayrton, A. D.; Clarke, S. E., Cytochrome P450 gene induction in rats ex vivo assessed by quantitative real-time reverse transcriptase-polymerase chain reaction (taqman). *Drug Metabolism and Disposition* **2006,** 34, (6), 1063-1069.

211. Pal, D.; Venkataraman, V., A portable battery-operated chip thermocycler based on induction heating. *Sensors and Actuators A-Physical* **2002,** 102, (1-2), 151-156.

212. Lao, Y.; Tay, F. E. H.; Xu, G.; Hartono, D.; Lee, Y. Y., A Non-Contact Micro Thermocycling Chip for Polymerase Chain Reactions *International Journal of Computational Engineering Science* **2003,** 4, (3), 651-654.

213. Huhmer, A. F. R.; Landers, J. P., Noncontact infrared-mediated thermocycling for effective polymerase chain reaction amplification of DNA in nanoliter volumes. *Analytical Chemistry* **2000,** 72, (21), 5507-5512.

214. Hoang, V. N.; Kaigala, G. V.; Atrazhev, A.; Pilarski, L. M.; Backhouse, C. J., Strategies for enhancing the speed and integration of microchip genetic amplification. *Electrophoresis* **2008,** 29, 4684-4694.

215. Fermer, C.; Nilsson, P.; Larhed, M., Microwave-assisted high-speed PCR. *European Journal of Pharmaceutical Sciences* **2003,** 18, (2), 129-132.

216. Chen, L.; Manz, A.; Day, P. J. R., Total nucleic acid analysis integrated on microfluidic devices. *Lab on a Chip* **2007,** 7, (11), 1413-1423.

217. Sambrook, J.; Russell, D. W., *Molecular cloning: A laboratory manual*. 3rd ed.; Cold Spring Harbor Laboratory Press: New York, **2001**; Vol. 1.

218. Stellwagen, N. C., Effect of the electric field on the apparent mobility of large DNA fragments in agarose gels. *Biopolymers* **1985,** 24, 2243-2255.

219. Littlewood, P.; Mitchell, V., 16S rDNA PCR Amplfication. *Technical Note* **2007**.

220. Roche Applied Science, FastStart Taq DNA Polymerase, 5 U/ul, Handbook. **2008**.

221. Promega, Nucleic acid amplification: Protocols and applications guide. **2009**.

222. Brivio, M.; Li, Y.; Ahlford, A.; Kjeldsen, B. G.; Reimers, J. L.; Bu, M.; Syvanen, A.-C.; Bang, D. D.; Wolff, A., A simple and efficient method for on-chip storage of reagents: Towards lab-on-a-chip systems for point-of-care DNA diagnostics. *MicroTAS 2007* **2007,** Paris, France, (2007), 59-61.

223. Kim, J.; Byun, D.; Mauk, M. G.; Bau, H. H., A disposable, self-contained PCR chip. *Lab on a Chip* **2009,** 9, 606-612.

224. Heap, D. M.; Herrmann, M. G.; Wittwer, C. T., PCR amplification using electrolytic resistance for heating and temperature monitoring. *Biotechniques* **2000,** 29, (5), 1006-1012.

225. Cepheid, SmartCycler® System Brochure. **2009**.

226. de Mello, A. J., DNA amplification: does 'small' really mean 'efficient'? *Lab on a Chip* **2001,** 1, (2), 24N-29N.

227. Jakeway, S. C.; De Mello, A. J.; Russell, E. L., Miniaturized total analysis systems for biological analysis. *Fresenius Journal of Analytical Chemistry* **2000,** 366, 525 – 539.