Optimisation of a microfluidic device for the pre-concentration and size separation of cell free foetal DNA from maternal plasma by capillary electrophoresis

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DNA

ABSTRACT

The discovery of cell free foetal DNA (cffDNA) in 1997 allows for the combination of accuracy as well as non-invasiveness for prenatal diagnosis. This non-invasive genetic test requires only a maternal blood sample from which the cffDNA can be isolated and analysed. In this work cffDNA was isolated from a maternal blood sample using a micro-fluidic device which was fabricated using hot embossing and laser ablation techniques. The DNA sample was first pre-concentration by electrokinetic trapping (EKT) and then isotachophoresis (ITP). The concentrated sample was then separated by size using capillary electrophoresis (CE), all in a single device. All parameters and processes concerned with the micro-fluidic device were optimised sequentially. These parameters include both the chemical components as well as the physical processes which occur. The DNA used for the optimisation protocol was analysed using fluorescence spectroscopy, agarose gel electrophoresis as well as an Agilent Bioanalyser. The optimised protocol included a 9% acrylamide/pDMA matrix, 3 M N,N-dimethylurea as a denaturing agent, with tris based buffers for pre-concentration steps and 1X TBE (tris/borate/EDTA) buffer for capillary electrophoresis. The applied voltage of ITP was 300 V and CE was carried out at 180 V. The timing at which DNA was extracted from the device was kept at time = 60 s intervals. The optimised protocol was then used for real sample analysis and these samples were obtained from mothers pregnant with male foetuses. The DNA extracted from the micro-fluidic device was then analysed using real time PCR (RT-PCR) in order to distinguish which was maternal and which was foetal. This was carried out by amplification of male and general (present in male and female) genes respectively. RT-PCR results confirmed that only the male specific gene was amplified in initial samples exiting the device and it was thus successful in isolating cffDNA from a maternal plasma sample.

DECLARATION

I declare that *Optimisation of a microfluidic device for the pre-concentration and size separation of cell free foetal DNA from maternal plasma by capillary electrophoresis* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated or acknowledged as complete references.

Candice Rassie November 2012

Signed …….

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

ACRONYMS AND ABBREVIATIONS

PMMA	Poly(methyl methacrylate)
PDMS	Polydimethylsiloxane
DNA	Deoxyribonucleic Acid
cffDNA	Cell Free Foetal DNA
EKT	Electrokinetic Trapping
ITP	Isotachophoresis
CE	Capillary Electrophoresis
RT-PCR	Real Time Polymerase Chain Reaction
IMM	Institut für Mikrotechnik Mainz
APS	Ammonium Persulfate
TEMED	Tetramethylethylenediamine
Bis	N,N′-methylenebisacrylamide
pDMA	Poly(dimethylacrylamide)
pHEA	Poly(hydroxyethylacrylamide)
PVP	Polyvinylpyrrolidone
HEC	Hydroxyethylcellulose
HPC	Hydroxypropylcellulose

LE	Leading Electrolyte
TE	Terminating Electrolyte
TBE	Tris/Borate/EDTA
Tris	Tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetraacetic acid

CHAPTER 1

1.0 INTRODUCTION

1.1. Background

Microfabrication is an upcoming technique with applications in various fields of research. It has been found particularly useful lately in the field of science and medicine. The reason for this trend is the push towards quicker and easier technology for the early diagnosis and treatment of disease. Miniaturisation is the key towards achieving this technology using advances in microfabrication. Fabrication on this small scale can be achieved using techniques such as etching, lithography [1], embossing, bonding [2], thin film deposition, doping [3] and polishing. Microfabrication found most of its uses in micro-electronics by using a semiconductor, such as silicon, as a substrate [4]. This type of microfabrication is mostly used to produce electrical components, such as integrated circuits, by fabricating smaller and smaller transistors. A "prediction" of miniaturisation was made my Gordon Moore in 1965, when he stated that the number of transistors on integrated circuits would double every two years [5]. In essence this means that these circuits could half in size in the allotted time.

However, silicon is not optimal for application in the scientific or medical field and thus plastics have currently been used. Substrates such as PMMA (polymethyl methacrylate) [6], PDMS (polydimethylsiloxane) [7], and PC (polycarbonate) [8] plastics are quite common for

biological applications. This is due to the low cost of plastics as well as the fact that they are easy to handle and manipulate using simple techniques. These characteristics make it possible for mass production of plastic micro-fabricated devices and thus the added benefit of disposability. Disposable devices minimise issues such as clogging, sterilisation and drift and are popular in a large number of commercial enterprises already [9].

Using tools provided by advances in microfabrication, on cheap substrates such as plastics, it is possible to produce devices that are small, portable and easy to use for point of care diagnostics. One of the common applications is the development of microfluidic devices for DNA analysis and sequencing. This is done by incorporating capillary columns for DNA electrophoresis, into a micro-fluidic system. The development of devices such as these is important for patients who cannot afford expensive tests and experiments carried out at hospitals and laboratories. This makes it possible for them to obtain results in a matter of minutes instead of hours or even days. The speed and efficiency of micro-fluidic devices also allow for the miniaturisation of what is routinely large and bulky equipment.

DNA sequencing is the process of reading a sequence of nucleotide bases which make up the genome. The first method of DNA sequencing is known as the Sanger method and is based on electrophoresis, which separates DNA strands according to size. Gel electrophoresis involves the migration of DNA fragments through a matrix by applying a potential between two oppositely charged electrodes, thereby causing size separation of the DNA. Gel electrophoresis usually occurs in a slab of agarose or acrylamide gel, but another method exists which is known as capillary electrophoresis. In this case DNA separation occurs in a thin capillary column instead of a slab of gel. Instruments that carry out conventional

capillary electrophoresis are big and usually consist of long and big capillary columns. By taking advantage of microfabrication of plastics, it is now possible to incorporate capillary columns into tiny micro-fluidic devices for DNA separation.

The miniaturisation of this new technology has various parameters which require optimisation. Especially if more processes are incorporated into a single device i.e. for the pre-concentration of a DNA sample. In the current work the optimisation of such a device is carried out. Pre-concentration of DNA can be carried out using various techniques in integrated devices such as sweeping techniques [10], solid phase extraction technology [11], field amplified stacking [12], electrokinetic trapping [13] and isotachophoresis [14]. The pre-concentration in this case is carried out by two subsequent methods i.e. electrokinetic trapping and isotachophoresis. The concentrated DNA sample can then be separated by size using capillary electrophoresis.

These three processes are carried out on a DNA sample inserted into the microfluidic device. First the DNA is pre-concentrated to minimise sample size and then separated according to DNA fragment length by capillary electrophoresis. New micro-fabricated devices have to be reliable and reproducible before its application to real samples. Parameters to be optimised include physical as well as chemical variables, in order to produce a device that can be used to separate DNA efficiently, quickly and with high precision in real DNA samples. The application of such micro-fluidic devices can be described in section 1.2.

1.2. Problem Identification

DNA analysis is one of the key diagnostic tools for genetic testing these days. Thus there is always a driving force towards achieving better and simpler methods of analysing DNA. Genetic diseases such as sickle cell anaemia, Down syndrome, coeliac disease and cystic fibrosis all have serious side effects which require early diagnosis and treatment. Recognition of these diseases early on allow for much better disease management as well as mental preparation. Genetic diseases not only affect adults but also unborn babies. Current methods of carrying out prenatal genetic tests are mostly invasive and might even result in miscarriage. These methods include amniocentesis, cordocentesis [15], chorionic villus sampling and even ultrasounds [16], which give very little information about any genetic abnormalities. Thus it is very important to develop prenatal tests which are non-invasive to both the baby and mother, and at the same time give sufficient information about the foetus.

- This was made possible in 1997 when the existence of cell free foetal DNA was discovered in maternal blood [17]. These are short fragments of DNA that belong to the foetus, but are circulating in the blood stream of the pregnant mother during gestation. These fragments of DNA are characteristically short in length and can thus be separated from maternal DNA according to size. The only sample one would need is a tiny amount of maternal blood from which both the foetal and maternal DNA can be extracted and tested. The cell free foetal DNA can hereby be isolated from the other DNA via size separation and taken for further genetic testing. This allows for prenatal diagnostic tests to be carried out without any stress to mother and child.

Pregnancy related complications such as pre-eclampsia results in a higher concentration of cell free foetal DNA circulating in the mother's blood. Theoretically, the level of cell free foetal DNA can be monitored as an indication of this disease. Pre-eclampsia is a disease that results in high blood pressure, oedema, and sudden weight gain as well as many other symptoms [18]. This disease can be very dangerous to the health of the mother and currently, diagnosis of this disease can only be carried out at either the hospital or a doctor's office. The isolation and quantification of cell free foetal DNA can thereby be a key tool for the diagnosis of pre-eclampsia as well.

Taking advantage of the fact that cell free foetal DNA exists in fragments less than 300 bp and maternal more than 500 bp, it is possible to use a micro-fluidic device described previously in order to isolate cffDNA from a maternal plasma sample. This size separation can be carried out by using capillary electrophoresis. The cffDNA can then be sequenced in order to obtain genetic information, but it can also be distinguished from maternal DNA with real time PCR. The RT-PCR protocol can be adjusted to detect, amplify and quantify foetal and maternal genes respectively to differentiate between cffDNA and maternal DNA.

1.3. Objectives

In order to develop a micro-fluidic device which is easy, cheap, simple and requires a minimal sample to analyse, many components have to be considered. This microfluidic device will essentially be used for the size separation of cell free foetal DNA from a real maternal plasma sample. Thus the following objectives for this work have been derived:

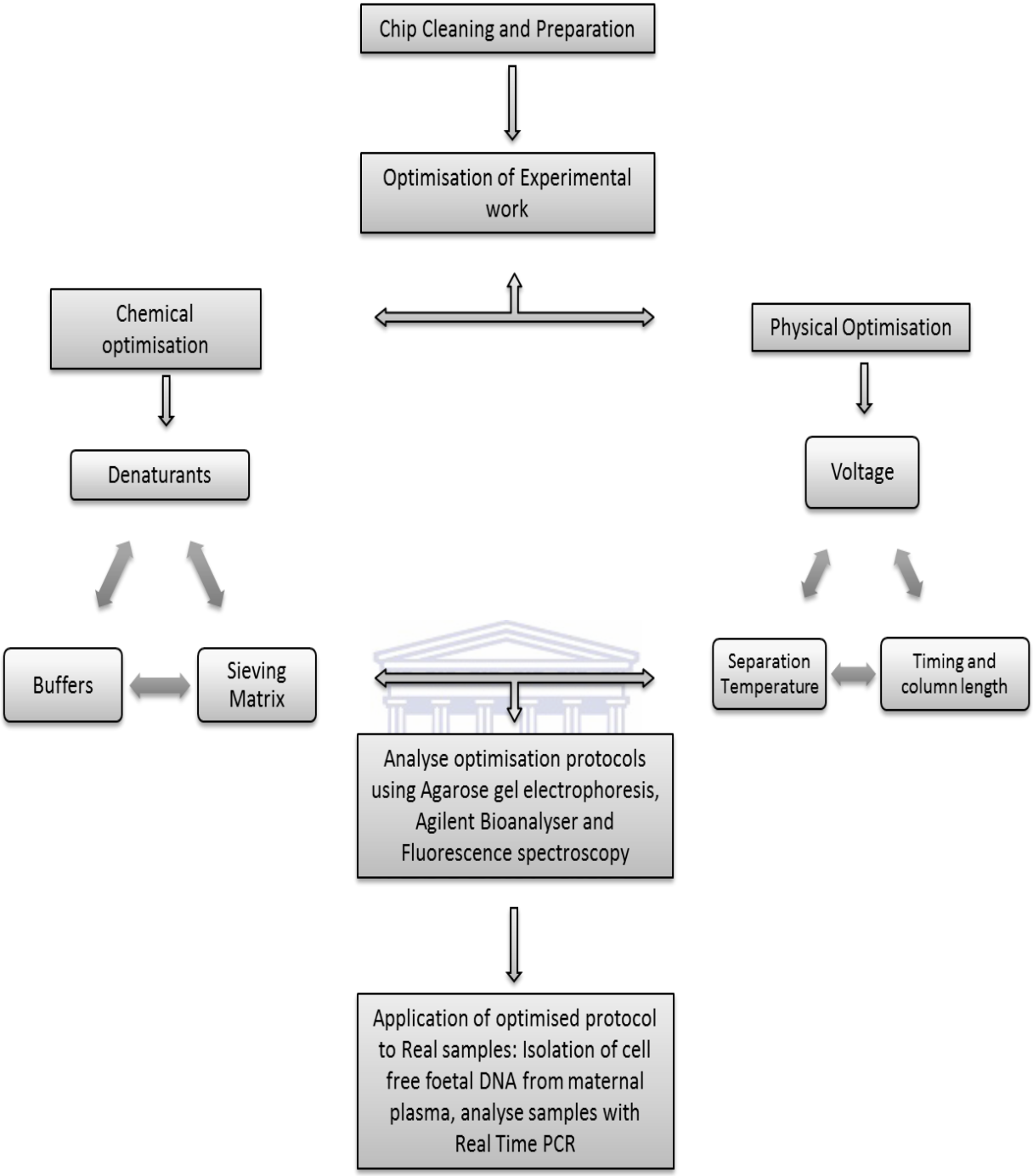
- To optimise chemical components of the micro-fluidic device including:
    - The buffers used within the micro-fluidic device
    - The sieving matrix in which DNA will separate according to size
    - The denaturing agent within the sieving matrix that increases resolution and separation of DNA fragments

- To optimise physical processes occurring within the microfluidic device
    - The voltage of electrokinetic trapping, isotachophoresis and capillary electrophoresis
    - The timing at which DNA is extracted from the micro-fluidic device for analysis
    - The temperature of capillary electrophoresis
    - The length of the capillary column

- To use the optimised micro-fluidic device and processes to separate cell free foetal DNA from maternal plasma samples

1.4. Research Framework

This research work followed a specific route in which various components have been optimised. Initially, the micro-fluidic device or chip was cleaned and prepared for the experiment. The optimisation of the experimental protocol can be separated into two different fields but were carried out as individual steps. These two fields are the chemical optimisation and the physical optimisation of the process. The chemical components optimised include the composition of the sieving matrix within the capillary column, the denaturing agent present in the column as well as the buffer system which exists in the chip. The physical processes to be optimised include the respective voltage of each step in the micro-fluidic device, the temperature of DNA separation, the timing of the overall process as well as the length of the capillary column. The optimised process will then be applied for the separation of cell free foetal DNA from a real maternal sample. The research framework can be represented by scheme 1:



Scheme 1: Research Framework

1.5. Outline of thesis

This thesis is divided into 7 chapters which will all be described briefly below:

Chapter 1 is the introduction which gives a brief background into the basic methods of micro-fabrication. The use of micro-fabrication to produce devices such as micro-fluidic chips, which are widely used in DNA sequencing and analysis, is discussed. It compares previous methods of prenatal diagnosis to the discovery of cell free foetal DNA in maternal plasma. This chapter also states the objectives of the work and also a framework of how the experimental process occurred.

Chapter 2 is a literature review relating to current methods of carrying out prenatal diagnosis and the difficulties associated with them. It discusses recent advances in prenatal diagnosis since the discovery of cell free foetal DNA. Methods of fabricating micro-fluidic devices are also briefly described. Furthermore the processes of pre-concentrating DNA such as electrokinetic trapping and isotachophoresis were reviewed. Capillary electrophoresis, which is the new way of sequencing and separating DNA according to size, has also been studied. Recent advances in lab on a chip technology are also discussed in this chapter.

Chapter 3 is the section concerning the techniques used in this work and is described in detail here. These techniques include hot embossing, laser microfabrication, electrokinetic trapping, isotachophoresis, agarose gel electrophoresis, capillary electrophoresis, fluorescence spectroscopy and real time PCR.

Chapter 4 contains a list of all the materials and instruments used, which is the first section explaining the experimental procedures. The protocols for assembling devices as well as the experiments carried out on DNA by these devices. The optimisation steps in the protocol are listed here as well as a basic statement about what these steps entail.

Chapter 5 goes into more detail about each optimisation step and what the result of each step entails. It also describes how the optimised protocol is applied to real samples as well as the real time PCR protocol used.

Chapter 6 contains all results concerning the optimisation process which are discussed in this chapter. Here, the result of this devices application to real samples is described.

Chapter 7 contains the conclusion of the entire work and recommendations for its future use are described here.

CHAPTER 2

2.0. LITERATURE REVIEW

2.1. Prenatal Diagnosis

Prenatal diagnosis is a key factor when carrying an unborn child, not only to monitor the baby but also for early detection of possible diseases or abnormalities. This is important to allow the mother the choice of treatment or to mentally prepare herself for the arrival of her baby. Various methods used for prenatal diagnosis are discussed below.

2.1.1 Non-invasive methods

Currently, there are a number of options when it comes to prenatal diagnosis. One of the most common methods of non-invasive monitoring of a foetus is the use of ultrasounds [19]. Ultrasounds date back to the early 19th century and have been a key tool for the detection and monitoring of congenital diseases. Diseases concerning the digestive system such as the gastrointestinal tract have been found to be detectable using ultrasounds [20]. This allows for management of these diseases in adults as well as being capable of monitoring foetuses. Congenital diseases such as cystic fibrosis [21], cleft palate [22] and even Down syndrome [16] have been detected using various forms of sonography. In one instance of cleft palate detection, a 3D mode of ultrasounds was used in which different planes are reconstructed to

form a multi-dimensional image. In another case of Down syndrome detection a nuchal translucency scan was done in order to monitor the thickness of the nuchal fold (neck). The presence of this increased nuchal thickness was found as an indication of Down syndrome in 1985 [23]. Another option of obtaining scanned images of a foetus is the use of foetal MRI (magnetic resonance imaging) which gives a higher resolution image of the internal organs of the foetus [24]. This method has been proven to detect and aid in the management of abdominal diseases in foetuses.

2.1.2 Invasive methods

Other forms of prenatal diagnosis are considerably more invasive than the scans described above. In the case of genetic testing, a well-known method of prenatal diagnosis is amniocentesis. This method entails the insertion of a needle into the amniotic sac and the withdrawal of amniotic fluid which undergoes testing for the detection of various diseases. However, the method is not only painful but has been found to lead to both foetal and maternal complications. Amniocentesis has previously been associated with miscarriages [15], amniotic band syndrome [25] and intra-amniotic infection which are possible risks of this technique.

An alternative to amniocentesis is chorionic villus sampling. In this technique instead of amniotic fluid being withdrawn, a sample of the chorionic villi is extracted i.e. placental tissue. Studies have shown that this technique has an even higher pain sensation than amniocentesis [26]. However, it has also been proven to give more reliable data as well as

allowing for rapid karyotyping, biochemical and genetic studies in early pregnancy compared to amniocentesis [27].

Along with the techniques listed above, there are other methods of prenatal diagnosis such as percutaneous umbilical blood sampling (PUBS), which involves the extraction and analysis of umbilical cord blood. A needle is inserted into the umbilical cord with the guidance of an ultrasound. The danger of this test however grows with the amount of punctures the umbilical cord undergoes. Foetal and neonatal deaths can be as high as 5% after this procedure [28]. Another invasive prenatal diagnostic test exists although it is not as commonly used. This test is known as an embryoscopy or foetoscopy. This technique can be used as both a diagnostic tool as well as a treatment option. It entails a small incision being made in the abdomen to allow an endoscope access to the amniotic sac, the foetus, umbilical cord and foetal side of the placenta. An endoscope inserted through the abdomen and uterus has previously been used to monitor abnormalities in membrane rupture and was proven successful [29]. This technique can also be used to treat birth defects such as tumours in vivo. A foetoscopic surgery was carried out in utero and was successful in removing all traces of an oral teratoma in an unborn baby [30]. However, in order to minimise the invasiveness of a test and maintain the high level of genetic and biochemical information obtained from it, a new method of prenatal diagnosis is needed.

2.2. Cell free foetal DNA

The discovery of cell free foetal DNA in 1997 revolutionised prenatal diagnosis [17]. This type of DNA exists as tiny fragments of DNA that belong to the foetus, but are circulating in the blood system of the mother from around 4 weeks gestation [31]. Although the exact source of the DNA was not previously known, it was suggested that they came from the connection which exists between mother and child through the umbilical cord [32]. However, more recently it has been proven that they emanate from trophoblast cells present in the placenta [33]. Analysis on cffDNA has shown that the whole foetal genome is present in maternal plasma [34], making it possible to monitor any kind of genetic disease that the foetus might have, from a sample of maternal blood.

2.2.1. Applications of cell free foetal DNA

Since its discovery it has found a wide application in prenatal diagnosis. These include sex determination [35], in which one group used quantitative fluorescent polymerase chain reaction (QF-PCR) to analyse cffDNA in first trimester maternal plasma. They were found to successfully determine the gender of the foetus using this method. In another study, quantitative PCR was used to determine the sex of a foetus from a maternal blood sample by amplification of the SRY gene. This gene is male specific and would thus only be amplified or have a positive signal in male foetuses. The protocol was found to have 97.2% sensitivity and 100% specificity [36] in this case. The results obtained from the groups study is shown in table 1 below:

Table 1: cffDNA results (Rijnders, R *et al*.)

SRY Real-Time Quantitative Polymerase Chain Reaction Results Obtained With Maternal Plasma in Comparison With Sex After Birth and/or Sex as Determined by Ultrasound.

Foetal sex	**21 Samples with 1 DNA isolation, number of positive replicates (interpretation of test results)**				**44 Samples with 2 DNA isolations, number of positive replicates (interpretation of test results)**			
	0/3 (negative)	1/3 (negative)	2/3 (positive)	3/3 (positive)	0/4 (negative)	1/4 (negative)	3/4 (positive)	4/4 (positive)
Female (*n* =29)	**10**	**1**	7†	3	**17***	**1**	7	19
Male (*n* =36)								

For the first 21 cases, 1 DNA isolation was tested (in triplicate), and for the subsequent 44 cases, 2 DNA isolations were tested (in duplicate).

** In 1 case, only 3 replicates could be performed.*

† In 2 cases, only 2 of the 3 replicates could be performed.

Table 2 above represents a study in which 65 blood samples were analysed in order to determine the sex of the foetus. The SRY real-time quantitative PCR test showed a negative result for all of the 29 female foetuses (results represented by bold print in table 2). In 35 of 36 male foetuses, SRY was amplified in both DNA isolations which yielded a positive result. [36]

Cell free foetal DNA has also been found to detect genetic disorders that contain an abnormal number of chromosomes, known as aneuploidies. A disorder in which one of the sex chromosomes are absent in women and only a partial chromosome is present in a single copy is known as Turner syndrome. This genetic disorder has great promise in being detected by

cell free foetal DNA analysis. A group has used DNA microarrays for the detection of Turner syndrome, another aneuploidy called trisomy21 (Down Syndrome) as well as SRY gene detection. This technique is known as comparative genomic hybridization (CGH) analysis [37]. In this case the group analysed cell free foetal DNA extracted from the amniotic fluid, now called AF cffDNA.

Trisomy 21 or Down syndrome has been detected using cell free foetal DNA numerous times in research. This disease results in three copies of chromosome 21 instead of the normal two copies. Another example of prenatal diagnosis using cffDNA was that of Farina *et al*. [38]. This group isolated cffDNA from maternal serum samples at the second-trimester of pregnancy. A comparison was made between the levels of cffDNA in the second trimester of pregnancy with Down syndrome pregnancy compared to unaffected ones. Results showed that Down syndrome cases had a 1.7 times higher level of cffDNA than normal and that this type of analysis can increase the screening performance of the disease in second trimester.

Single gene disorders are the results of a single gene mutation and cause various serious diseases such as Huntington's disease, cystic fibrosis and also sickle cell disease. As we know, all these diseases have severe side effects which have a negative impact on the quality of life and are thereby important to diagnose prenatally. This has been proven possible by analysis of cell free foetal DNA by the Department of Genetics in Madrid, Spain. This group managed to non-invasively diagnose Huntington's disease in foetuses whose fathers were affected, using cffDNA [39]. This group also used quantitative fluorescent polymerase chain reaction (QF-PCR) to detect this particular genetic disorder from a maternal plasma sample. Usually Huntington's disease is diagnosed prenatally using chorionic villus sampling which

poses a 0.5%-1% risk of abortion. Gonzales-Gonzales *et al*. [40] has improved on methods of non-invasive diagnosis of this disease in foetuses. This group used cffDNA sequences of paternal origin to analyse and diagnose the disease, since the maternal DNA in plasma caused a large interference. Cell free foetal DNA has also been used to detect other gene disorders such as cystic fibrosis by the same group in 2002. Using a similar protocol as with Huntington's disease, the group successfully detected the paternally inherited mutation at 13 weeks gestation [41]. This analysis was carried out on cffDNA isolated from maternal plasma and serum samples.

The uses and applications of cell free foetal DNA has successfully shown great promise as a non-invasive tool for prenatal diagnosis and has a definite future for commercialisation of these genetic tests. If it is possible to produce a device that is inexpensive, easy to use and portable, cffDNA can further revolutionise prenatal diagnosis. It is possible to take advantage of recent advances and the ease of microfabrication to possibly manufacture such devices for public use.

2.3. Microfabrication

Microfabrication found its origin in electronics, with silicon as a substrate. This semi-conductor has been widely used for the development of electronic circuits. However, microfabrication has also found a large application in both biology and medicine. These applications are generally divided into four classes: tools for cell biology, tools for molecular biology, biochemistry and medical devices [9]. The application of analysing cffDNA would

ultimately fall into the category of becoming a medical device in the case of this work. Although not a device used for treatment, it would have diagnostic capabilities. The application of biological devices such as these has grown much more in the last decade, due to demands such as commercialisation of products, low volume consumption technologies and high throughput requirements. One of the substrates which have the capabilities of fulfilling these requirements is plastic. This is due to the fact that plastics are easily obtainable and very cheap; they are also easily manipulated into specific designs, as well as the fact that they can be mass produced easily. Various methods of micro-fabrication of plastics exist such as injection molding [42], casting [43], hot embossing and even laser ablation. The last two techniques mentioned will be reviewed in more detail since they were applied in this research work.

2.3.1. Hot embossing

Hot embossing is a common technique for the production of micro-devices using thermoplastics. This technique requires a mold or stamp from which it gets its structure and form. The most common stamp or mold used is made of polydimethylsiloxane (PDMS). The reason for its use is the elastic properties of PDMS as well as the low surface energy of the polymer, which makes it easier to demold after the hot embossing process. Goral *et al* successfully produced simple micro-fluidic devices using a PDMS mold and a polystyrene substrate [44]. The products in this case were micro-fluidic devices with large structures. The PDMS molds could be re-used in order to make further replicas of the device.

The process of hot embossing is shown in Figure 1 below. In a study by Becker *et al*., hot embossing procedure included the production of the embossing master by the LIGA method (LIGA is a German acronym for lithography, electroplating and molding – Lithographie, Galvanik, Abformung). The master was then used to emboss a pattern into a thermoplastic polymer i.e. either PMMA or PC. The master and substrate is placed onto the heating plates of the equipment. In the beginning of the procedure both are heated separately in a vacuum chamber. For these thermoplastics, this temperature is in the range of temp = 100-180 ^{0}C. After this the two parts are brought together with a force of 20-30 kN. The combined parts are then cooled to just below the glass transition temperature of the polymer and after some time the two are pulled apart. This method has been shown to results in high aspect ratio micro-structures in both polymers [45].

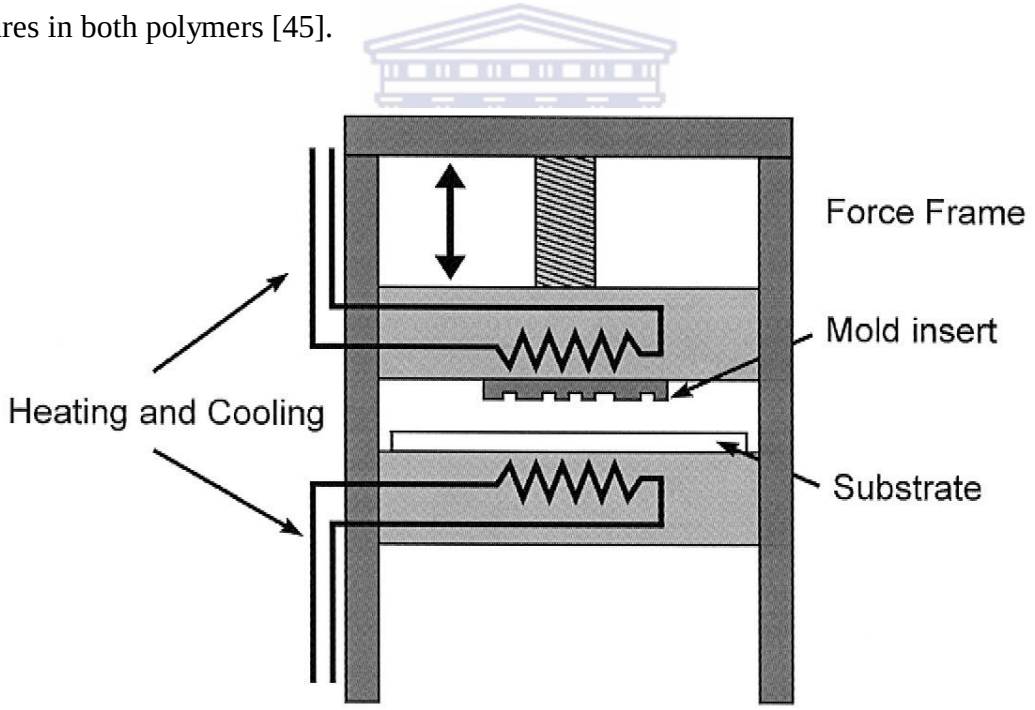


Figure 1: Schematic drawing of the hot embossing equipment

Hot embossing is mostly used for devices involving capillary electrophoresis, but is also used to fabricate more complex structures and devices such as actuators. An actuator produced by one group was used to control the flow of paraffin in a particular device [46]. A

polycarbonate substrate was hot embossed with a silicon master electroplated with nickel by Klintberg *et al*.

The three heating and cooling processes involved in hot embossing can be represented in Figure 2 below:

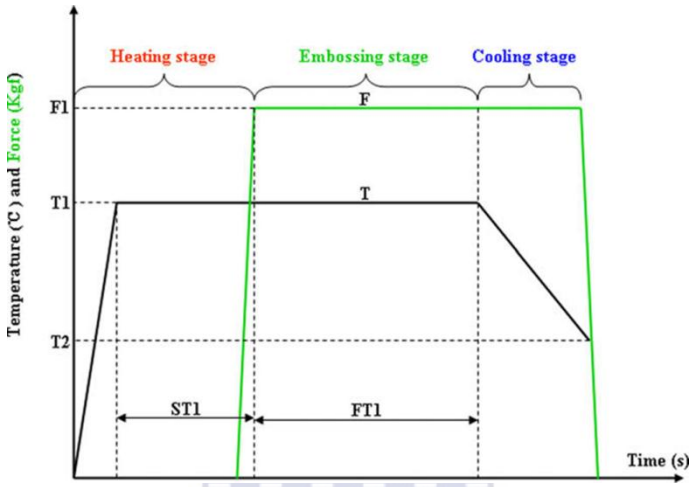


Figure 2: Three stages of hot embossing

[47]

As stated before, hot embossing involves heating up to the glass transition temperature of the polymer, an embossing of the pattern into the polymer and the cooling and separating of the polymer from the mold. Hot embossing is thus a viable tool for the mass production of devices such as the one described in this work, and its use in the isolation of cell free foetal DNA.

2.3.2. Laser Ablation

The second method of microfabrication used in this work is known as laser ablation. This process involves the irradiation of solid material from a surface with the use of a laser beam.

Microfabrication of plastics is usually carried out using carbon dioxide (CO_2) or excimer lasers [48]. Carbon dioxide lasers operate in the infrared region of the electromagnetic spectrum whereas excimer lasers operate in the UV range. Laser ablation usually removes material with a pulsed laser, but continuous wave lasers also exist. The pulse of the laser is what measures the speed and can vary in duration from milliseconds to femtoseconds.

The most commonly used substrate for microfabrication with CO_2 lasers is PMMA and PET [49]. This is because of the ease of control as well as the capabilities of PMMA to subsequent bonding methods. Other substrates have also been ablated by excimer lasers, such as polycarbonate (PC), polyimide, polyvinyl chloride (PVC), cellulose acetate and polystyrene (PS) [50]. As stated before, PMMA is one of the main substrates for CO_2 lasers. For the purpose of this work, I will focus on this topic for review. PMMA plastic is commonly used as a substrate for the production of micro-capillaries for DNA electrophoresis. Laser fabrication of these devices allows for rapid, precise and reproducible products. Pfleging *et al.* was able to produce capillaries of less than 30 μm in width with an average deviation of around 3% [51]. The laser used had a continuous mode with a maximum beam power = 40 W.

The fact that direct-writing of a CO_2 laser is fast has the negative side-effect of forming a rugged surface on substrates. This in turn results in a surface which has limited chemistry modification ability. However PMMA micromachining was shown to improve on these effects in a work by Cheng *et al.* [52]. A commercial laser scriber was used to design and engrave patterns onto the PMMA substrate. In order to overcome the surface roughness, a thermal annealing process was included to smoothen out the PMMA structures. Because of

the chemical characteristics of PMMA, it was possible to further modify the surface of the plastic. This was performed by a reduction reaction followed by organosilane chemistry. These reactions resulted in the introduction of functional groups such as perfluoroalkyl (–C_nF_{2n+2}), amino (–NH_2), and sulfhydryl (–SH) groups. These functional groups allow for surface passivation or further biomolecule immobilization. It has thus been sufficiently proven that CO_2 lasers are suitable for the micro-fabrication of PMMA substrates in the development of biological devices.

2.4. Capillary electrophoresis

Capillary electrophoresis is considered an evolution of regular gel electrophoresis. In general terms, the equipment consists of an injection system, a separation capillary, a high voltage source, electrodes, electrode jars as well as detectors for the separation and analysis of molecules such as DNA. There are various separation techniques which exist for a capillary electropherograph. These include capillary zone electrophoresis, micellar electrokinetic capillary electrophoresis, capillary electrochromatography, capillary isotachophoresis, capillary gel electrophoresis, capillary isoelectric focusing and chiral separation methods [53].

Capillary electrophoresis is one of the most commonly used techniques for DNA sequencing in recent years and has been widely used in the area of DNA analysis. DNA sequencing efficiency and accuracy using capillary electrophoresis is dependent on various factors such as the electric field strength, temperature, composition of the sieving matrices as well as capillary wall coating, DNA labelling and DNA sequencing performance [54]. For the

purpose of this work, we will consider only the parameters which affect the micro-fluidic device used.

2.4.1. Effect of various parameters in capillary electrophoresis

The sieving matrix which fills the capillary column largely effects the migration of molecules within the capillary. This has an influence on both experimental time as well as molecule resolution. Initially, cross-linked polyacrylamide matrices were used. However this matrix has a very limited lifetime and is not suitable for repeated use [55]. The cross-linked polymer was then replaced by a more robust linear form of acrylamide (LPA/linear polyacrylamide). This matrix has the added benefit of being replaceable in the capillary column [56]. Derivatives of acrylamide such as poly(dimethylacrylamide) or pDMA has also found application in CE. The fact that this polymer greatly reduces electro-osmotic flow (EOF) makes it advantageous as a sieving matrix. Another extensively used polymer in capillary electrophoresis is poly(ethylene oxide) (PEO). This polymer has the ability to dynamically coat capillary walls, has a low viscosity and thus requires a low pressure for filling of capillaries [57]. Cellulose based polymers such as hydroxyethylcellulose and hydroxypropylcellulose has also been used for separation of molecules such as DNA [58]. An ideal or universal polymer for all sizes of DNA sequencing or separation has not yet been found. Some polymers might be ideal for shorter fragments while others are better for longer fragments. Others might have a better read length while others have ideal viscosity. Thus it is important to optimise the sieving matrix specifically for the application on which it is used.

The sieving matrix generally contains a denaturing agent which improves the separation of different sized DNA fragments in capillary electrophoresis. The most common denaturing agent for gel electrophoresis is urea and sometimes derivatives thereof such as N,N-dimethylurea and tetramethylurea [59]. Organic solvents have been used more commonly as a denaturing agent in capillary electrophoresis. Rocheleau *et al*. has made use of a 10% formamide content to improve denaturing ability of the sieving matrix [60]. Other denaturing agents include N-methylformamide [61] as well as 2-pyrrolidinone [62].

The effect of temperature has also been shown to act somewhat as a denaturing agent. The increase in temperature to around 60 ^{0}C has been shown to improve resolution and separation efficiency in capillary electrophoresis [63].

The voltage or electric field strength has an impact on read time as well as separation and read length. In this case a high voltage would speed up the migration time of DNA but have a negative impact on fragment separation and vice versa for a low voltage. A group at Iowa State University showed the positive effect of a low separation voltage to have excellent resolution of DNA [64]. The effect of this low voltage however extended the electrophoresis time to 7 h. Increasing the electric voltage from 75 V/cm to 600 V/cm reduced this to time = 3-4 min, in the work of Muller *et al.* [65]. However the downside of this is that the read length dramatically decreased. Thus it is important to optimise this factor as well for every application used.

2.4.2. Applications of capillary electrophoresis

Although capillary electrophoresis is widely and most commonly used for sequencing of DNA, it has numerous other applications. Capillary electrophoresis has found an application in forensics and toxicology. This was done by the investigation of illicit drugs in seized preparations as well as complex biological matrices. CE was used by a group to simultaneously differentiate between various drugs from a hair sample in 1995 [66]. Another group used capillary electrophoresis for the separation of over 50 metabolites from a urine sample. Generally, this clinical technique would be carried out using GC-MS (gas chromatography and mass spectrometry), but the use of CE reduces the time taken from over an hour to just 15 min [67]. Capillary electrophoresis can also be used in numerous clinical laboratory applications such as analysis of proteins, haemoglobin variants, lipoproteins, molecular diagnostics and many others [68].

2.5. DNA pre-concentration

There are various means of on-chip pre-concentration of DNA samples in micro-fluidic devices. These include techniques such as field amplified sample stacking (FASS), temperature gradient focusing, electrokinetic trapping (EKT) and isotachophoresis (ITP). For the purpose of this work the last two techniques will be reviewed i.e. EKT and ITP. It is possible to work with larger sample volumes using these techniques and therefore they were applied in this research work.

2.5.1 Electrokinetic Trapping

Electrokinetic methods are one of the most popular techniques of pre-concentrating DNA in micro-fluidic devices. This is due to the possibility and advantage of using samples with a larger volume of a few microliters. EKT phenomena has been reported and explained by the hindrance of charged molecules (such as DNA) which enter channels or nano-sized pores. This hindrance is caused by charge exclusion [69]. Electrokinetic phenomena can occur in front of many other surfaces such as hydrogel plugs, nano-channels as well as membranes. A negatively charged hydrogel plug was successfully used within a channel in order to concentrate negatively charged analytes [70]. The charged analytes were concentrated in front of the hydrogel which contained pores of only a few nanometres (~20 nm). However this phenomenon of pre-concentration was found to be affected by a major redistribution of the local electric field. This was caused by development of CP (concentration polarization) zones and simultaneous generation of electro osmotic flow (EOF) that hindered pre-concentration.

Electrokinetic interaction of charged or neutral molecules has also been studied in various depths of nano-channels. A group investigated the electrophoretic transport of 100-1000 bp DNA fragments in a fused silica channel of depth = 40, 100 and 1560 nm. This channel contained no sieving matrix or gel [71]. This group however showed that the electrokinetically-driven DNA separation in nano-channels needs further improvement in order to make accurate predictions. It is suggested that the surface of these nano-channels as well as the kinetics of adsorption/desorption play a big role in nano-channel electrophoresis [72].

Electrokinetic trapping in front of a membrane is particularly interesting for this work. This technique has used membranes with pores in the range of 2-200 nm in diameter. This size is larger than the sample molecule and thus disallows mechanical trapping. A polarization zone forms, in which the specific conductivity increases on the cathodic side of a cation-selective membrane. This zone causes the sample ions to decelerate and thereby concentrate in front of the membrane [73]. The use of poly(ethylene terephthalate) as a membrane for electrokinetic trapping has been proven successful in the past. This polymer provides a negatively charged surface [74] so as to exclude negatively charged molecules such as DNA. A microsystem was recently fabricated so as to incorporate this negatively charged PET membrane for the electrokinetic trapping and thereby pre-concentration of a DNA sample [75]. The use of electrokinetic trapping of DNA in front of a negatively charged membrane is thus a viable option for pre-concentration of a sample of a few microliters.

2.5.2 Isotachophoresis

Isotachophoresis (ITP) is a technique of concentrating or separating analytes which is applicable to many different samples. These include peptides [76], small ions [77], nanoparticles [78], pharmaceuticals [79] and in this case DNA. It is possible to analyse so many molecules using this technique because it is mostly dependent on a gradient within the buffer system and not so much a specific trait of the analyte. The buffer system consists of a leading (LE buffer) and terminating (TE buffer) electrolyte which contains fast and slow ions respectively, causing analytes to focus at the interface of the two under the influence of an applied electric field.

The application of this technique varies across a wide spectrum of analytes. A group, Prest *et al.* [80] used a complexing agent (malonic acid) in conjunction with isotachophoresis to concentrate and thereby quantify magnesium in a sample with many other analytes. The technique resulted in a limit of detection of 0.45 mg/L using 10 mM caesium hydroxide + pivalic acid, as LE buffer, 2 mM malonic acid, 1 g/L HEC as a sieving matrix and 10 mM tris as a TE buffer. Another group uses HEC at 0.08% concentration in the determination of neomycin. This group carried out ITP using a column switching commercial apparatus with conductivity detection. In this case the LE buffer consisted of 10 mM sodium acetate-acetic acid, HEC and TE buffer of 10 mM b-alanine. A limit of detection of 18.96 mg/L was achieved using this technique [81].

A type of isotachophoresis that takes place at the beginning of a capillary electrophoresis column is known as transient isotachophoresis or tITP. A group has previously combined electrokinetic trapping and isotachophoresis in a single-channel microchip for analysis of DNA [82]. This group employed a single channel with length = 40.5 mm, width = 110 micron and depth = 50 micron. They found that using a focused electrokinetic injection and transient isotachophoresis as a pre-concentration protocol greatly improved the sensitivity of DNA analysis by chip electrophoresis. The group attempted to further improve on this work by using a different geometry of the micro-chip. A novel sample injection system was proposed and named floating electrokinetic supercharging. Here electrokinetic injection and ITP pre-concentration was performed in a separation channel. This channel connected two reservoir ports with a cross-geometry micro-chip. A representation of the chip geometry is as shown in Figure 3 below:

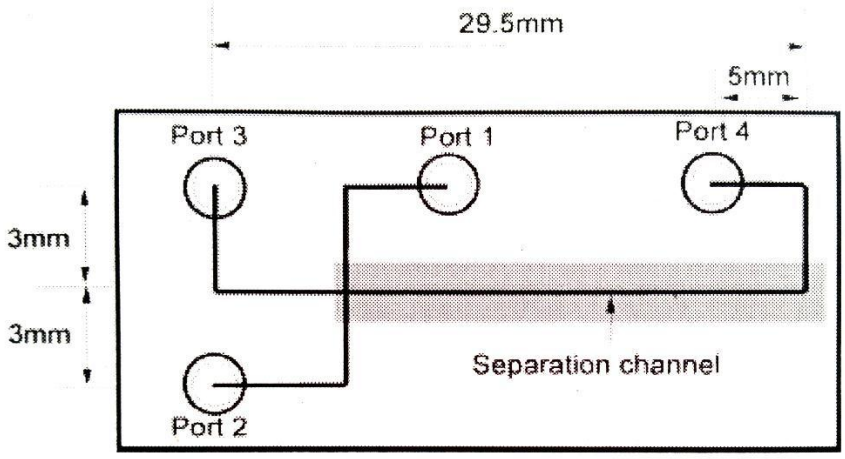


Figure 3: Schematic drawing of cross-geometry microchip. Ports 1, 2, 3 and 4 are reservoirs

The sample was eluted after pre-concentration using leading electrolyte ions in order to enable zone electrophoresis. This method allowed for a ten time increase in limit of detection as well as a higher resolution and shorter analysis time [83]. Isotachophoresis carried out in solution before the analyte enters the sieving matrix is known as free-solution transient isotachophoresis (FstITP) when coupled with capillary electrophoresis. The group found that the pre-concentration step in solution was much more rapid in free solution which resulted in higher separation efficiency in the subsequent CE step. This FstITP-CE method was found to be suitable for DNA samples with a high saline content and is also simple, robust and flexible [84].

2.6. Lab on a Chip

The incorporation of two or more of these laboratory techniques into a single device is the definition of lab on a chip. The demand for this technology increased in recent years because

of the need for faster, smaller and more efficient analytical techniques. Two technologies arose from this demand, the first being selective chemical sensors and the second being total analysis systems (TAS). The latter is considered lab on a chip technology. A TAS device ideally contains all the components necessary for automated analysis such as sampling, sample pre-treatment, chemical reactions, analytical separations, analyte detection and product isolation as well as data analysis [85]. Manz *et al*. first introduced the concept of micro-total analysis systems in 1990 as an alternative method for chemical sensing. This work discussed theories of hydrodynamics and diffusion which indicated that both faster and more efficient processes could occur in flow injection analysis, chromatography and electrophoresis in a micro-TAS. He stated that the smaller sample volumes will improve selectivity and also that the device allows for a multi-component system [86].

Lab on a chip (LOC) technology has various applications, as with any laboratory technique. LOC devices also have the added benefit of being tailor made and designed for any specific application. Devices have been successfully produced for the analysis or diagnosis of plant pathogens. A group developed a system for the rapid determination of nucleic acids specific for the *Phytophthora* species. This device consisted of two components, flexible and non-flexible, with integrated micro-channels and micro-chambers. This device was capable of carrying out PCR steps and hybridisation of DNA sequences consecutively within a single device [87]. Another group took advantage of spectrophotometric detection for the characterisation of porcine oocytes. This micro-device consisted of micro-channels within a silicon substrate, buffers and optical fibres for detection of the oocyte as shown in Figure 4 below:

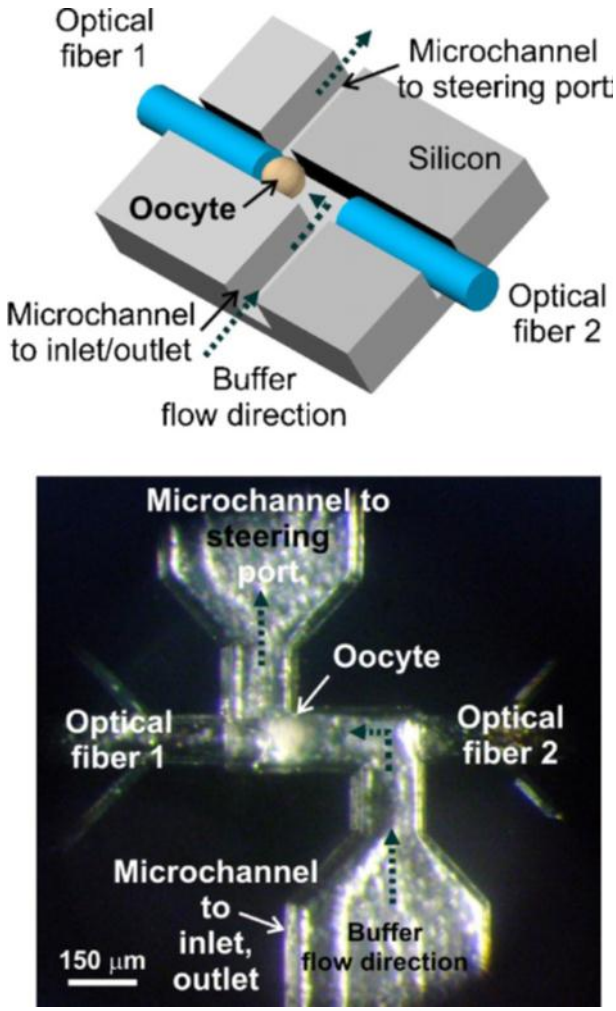


Figure 4: Images of the lab on a chip device for visualisation of a porcine oocyte

This group recorded the absorbance characteristics of the oocyte and stored it in a database. With this information in hand it was made possible to characterise and in future detect this cell [88] using the same device.

Lab on a chip is not only limited to analytical and characterisation techniques, but can also be used for synthesis of various structures or morphologies. In one instance, machining and hot-pressing of cyclic olefin copolymers were implemented to construct milli-fluidic LOC's which are able to carry out on-chip bubble generation and flow chemistries. This phenomenon

was then manipulated to generate porous solids that are highly mono-dispersed and ordered [89]. LOC technology has also expanded to the field of proteomics. Research has been carried out in order to enable protein sizing using lab on a chip technology and its possible integration into mass spectrometry equipment. Automated and rapid protein sizing have been available on chips in the current commercial market. Most groups make use of capillary electrophoresis as an alternative to mass spectrometry for protein sizing [90].

Microfabrication for on chip capillary electrophoresis of DNA is one of the most common techniques nowadays. A group was able to miniaturise an instrument for chip-based capillary electrophoresis of DNA with fluorometric detection. This device used a temperature control technique to change the mobility of DNA. Instead of the conventional plastic chips, this group made use of borosilicate glass substrates which contained micro-containers, a loading channel and separation channel for DNA analysis [91]. Lab on a chip technology in the form of DNA microarrays have also shown promise as an analytical tool or for the detection of cell free foetal DNA. Larrabee *et al*. [37] used comparative gene analysis by comparing the male specific SRY gene and X-chromosome markers for rapid screening of samples. In this way it is possible to carry out many experiments in parallel and obtain huge amounts of genetic information in a fraction of the time it would have taken using conventional DNA sequencing.

Cell free foetal DNA has been largely studied and efforts are constantly being made for the efficient and accurate separation and/or analysis of this DNA in order to carry out non-invasive prenatal diagnostic tests. It is thus natural that a lot of the studies are taking place on the micro-scale and have an application in LOC technology. Kubicki *et al.* has developed a glass LOC specifically for the size separation of cell free foetal DNA from a maternal sample.

In this case the group used synthetic DNA within the size range of foetal and maternal DNA and separated them within a borosilicate glass micro-fluidic device. The DNA was then detected using a laser and CCD camera for monitoring of fluorescent signals. They found that the DNA in a range of 20-500bp was separated in the device within time = 2 min, allowing for efficient extraction of DNA [92]. The work in this dissertation is an alternative to the technique described above which also employs size discrimination as a method of isolating cell free foetal DNA.

CHAPTER 3

3.0. METHODOLOGY

The fabrication of the micro-fluidic devices and analytical methods used in this experimental work are all described in detail below:

3.1. Hot Embossing

This method was used to fabricate the micro-fluidic device that contained the various components and reservoirs as can be seen in Figure 10 in section 4.4. Hot embossing is a form of micro-thermoforming of a substrate. In this case, a thermoplastic such as PMMA can be moulded into a specific shape with the help of a stamp as well as an increase in temperature. The stamp and substrate are placed into holders within the embossing equipment. The two pieces are then heated to a temperature just above the glass transition, T_g, of the polymer. The glass transition temperature is a thermal transition that a polymer undergoes where it softens because of the onset of long range molecular motion. This causes a change in the free volume of the polymer which can be represented by the graph below with a comparison of stiffness of polymer as a function of temperature in Figure 5 below, where T_m is the melting temperature of the polymer:

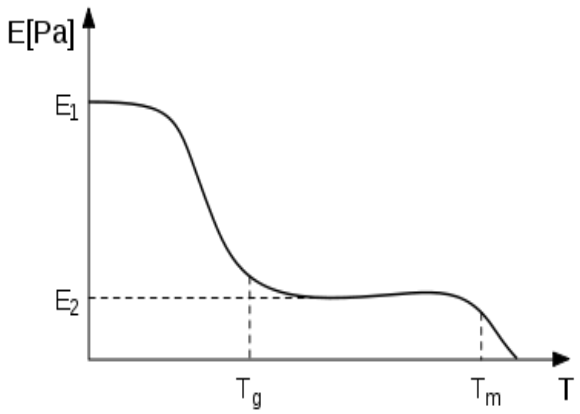
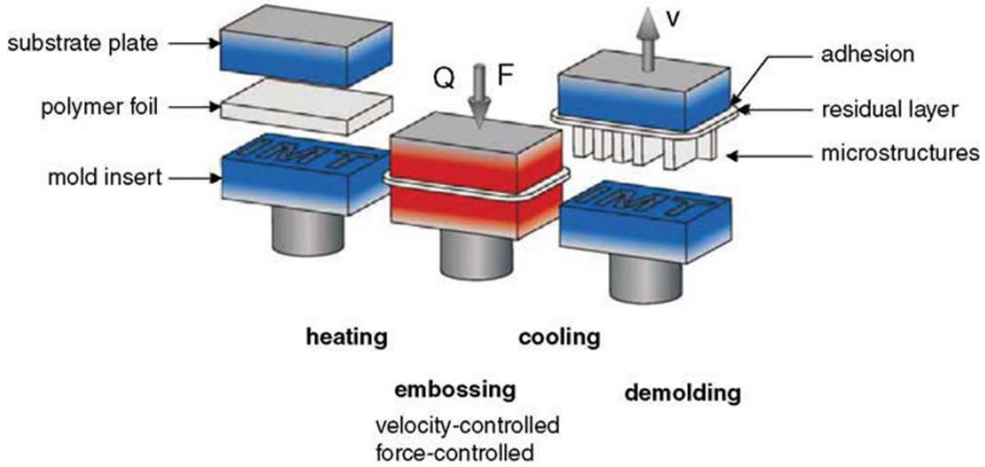


Figure 5: Graphical representation of glass transition temperature. (Stiffness versus temperature)

After this temperature is applied, the master and polymer substrate are brought into contact and pressed together under pressure and vacuum. This process occurs in two steps as a part of the molding cycle. The first step consists of the mold and polymer substrate moving toward each other at a speed of approximately 1 mm/min. This is done until the preset maximum embossing force is reached. This embossing force is kept constant for a period of time, known as the holding time which occurs in the second step of the molding cycle. In order to obtain a constant force between the two parts, the movement between tool and substrate has to be controlled. During the holding time, the heated polymer is allowed to flow in a radial direction while the remaining layer undergoes reduction under this applied force/pressure. At all stages in the holding time the temperature remains constant under vacuum. This is required in order to fill all the cavities of the mold completely as well as to eliminate the inclusion of air which might result in an incomplete filling of the mold. When the preset holding time is complete, the cooling step of the hot embossing protocol begins. This is continued until the temperature of the polymer substrate reaches just below its glass transition temperature. This can be called the demolding temperature i.e. the temperature at which the polymer substrate

and the mold are separated. For demolding to be successful, the adhesion between mold and substrate has to be less than the adhesion between substrate and the plate/holder. Because of these adhesive properties, the demolding occurs in such a way that it is both homogenous and vertical to the molded part. The process of demolding is the most critical for successful pattern transfer in hot embossing and can be influenced by a variety of factors. Inefficient demolding might even result in the destruction of the products microstructures [93]. A representation of the various processes is as shown in Scheme 2 below:



Scheme 2: Hot embossing Process

3.2. Laser Ablation/microfabrication

Laser ablation is another method with which to produce features of the micro-scale onto plastic substrates. Laser microfabrication can exist in three different forms or techniques, infrared laser micromachining, laser-induced micro-joining and femtosecond lasers.

The first type of laser microfabrication, infrared laser machining is based purely on thermal evaporation of material. This is carried out by applying a high heat in the form of a laser beam. This laser has an output wavelength in the infrared region of the electromagnetic spectrum. Laser micro-joining is mainly used as a method of welding together biocompatible components such as plastics to metal or glass parts. Laser welding of polymers can be performed using infrared radiation (with either an Nd: YAG laser, emitting at a wavelength = 1064 nm, or diode lasers emitting in a range of wavelength = 808 and 980 nm), which corresponds to a transmission window of the polymers. The transmission welding technique based on heating plastics via surface absorption can also be applied to produce a joint between two overlapping polymer substrates, one which acts as the transmitting and the other as the absorbing layer of plastic [94]. Femtosecond lasers can be used for laser ablation/microfabrication of plastics and its application to micro-devices. These lasers have the ability to produce light pulses of extremely short duration in the order of femtoseconds (time = 10^{-15} s). [95]. One of the common lasers which act at this speed pulse is the carbon dioxide laser.

Carbon dioxide lasers are one of the most efficient and powerful lasers operating in the middle infrared region. These can exist in both the pulsed and continuous wave forms. There are several different types of gas discharge configurations that consist of not only CO_2, but also nitrogen and helium. These lasers are able to produce powers of more than 100 kW with pulse energies of up to 10 kJ. This is because of the rotational-vibrational transitions of the CO_2 molecule. The laser can exist in one of three structures: Longitudinally excited lasers which are in the form of a long, narrow, cylindrically shaped glass enclosure with electrodes at opposite ends from which the discharge excitation current is introduced; waveguide lasers which have two transverse radio-frequency (RF) electrodes that are separated by insulating

sections which form a bore region. The bore region acts as a waveguide by reflecting the beam off the insulating material; and finally transversely excited lasers which operate at height total gas pressures of 1 atmosphere or more. This allows for a higher energy output per unit volume of gas. [96]

The laser beam is focused onto the surface of the substrate using parameters which are preset according to the specific requirements of the device, in this case a plastic micro-fluidic device. As the laser moves across the substrate surface, the polymer or plastic melts and some immediately forms a vapour, while the rest forms a pool of molten polymer on the surface. This pool moves away from the beam in all directions, driven by the heated gas of vaporised plastic. Most of the melted polymer re-solidifies, and in this way the laser beam is able to cut out channels and wells into a polymeric substrate. A schematic diagram of this process can be represented in Figure 6 below: [49]

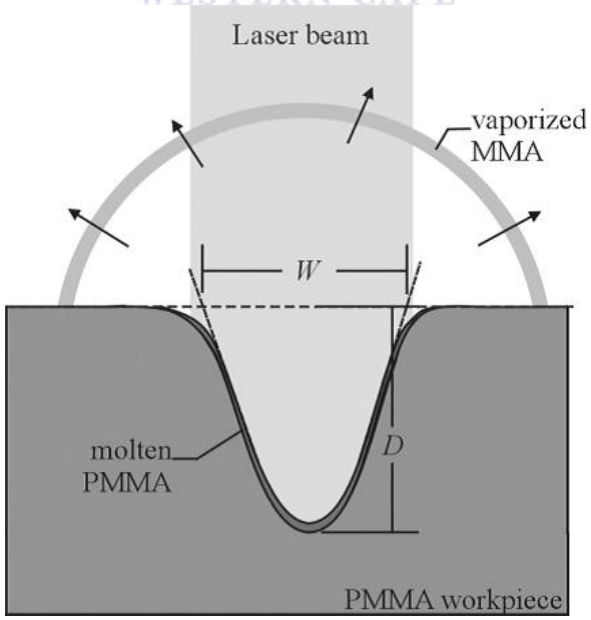


Figure 6: Micromachining by CO_2 laser

Both hot embossing and laser microfabrication can be used to make various micro-devices. In this work it was used to produce micro-fluidic devices for the pre-concentration and size separation of DNA and its application in isolating cell free foetal DNA. The processes occurring in the device will be reviewed in the following sections.

3.3. Electrokinetic Trapping

Conventional electrophoresis is based on the ratio of charge and size of the analyte particle. The electrophoretic force is directly proportional to the electric field applied as represented by the equation 1 below:

$$f_{ep} = zFE \qquad \textbf{Eq 1}$$

The electrophoretic force in this case is f_{ep}, z is the effective charge on the molecule, F is faradays constant and E is the electric field. [97]. Dielectrophoresis however refers to the effect of an applied electric field gradient on a polarizable particle. When this particle is more polarizable or charged than the surrounding medium the particle experiences positive dielectrophoresis and when the particle is less polarizable than the surrounding medium, the particle is repelled by the regions of high field gradient or negative dielectrophoresis. In our case this can be explained as electrokinetic trapping. The dielectrophoretic force on the particle can thus be expressed as the following equation 2

$$f_{dep} = 2\pi\epsilon_m r^3 K \nabla E^2 \qquad \textbf{Eq 2}$$

The dielectrophoretic force is thus represented by f_{dep}, m is the permittivity of the medium, r is the particle radius, E is the strength of the electric field and K is the Clausius-Mosotti factor. This value is dependent on the conductivities and permittivities of the medium and particle of the frequency of the applied field [98]. As shown, the dielectric force is proportional to the field gradient rather than the strength of the applied field and thus independent of the direction of the applied field. The dependence of frequency in K, the Clausius-Mosotti term, is due to the polarizability of dielectric particles dependence on the frequency of the applied field. The existence of this frequency is what allows particles such to either be trapped or repelled by the high field gradient by adjusting the frequency. This allows for dielectrophoresis trapping or pre-concentrating a particle of interest [99] [100].

Electrokinetic trapping in this case is a type of DNA concentration step occurring in front of a negatively charged membrane of poly(ethylene terephthalate) or PET. This membrane is characteristically negatively charged and placed in between a fluidic chamber and micro-channel. The fact that the PET membrane is permiselective induces a concentration polarization which exists near the channel. This occurs as an electric field is applied between an anodic or cathodic compartment and reservoir with the membrane connecting the two. The PET membrane consists of nanopores which have a diameter = 100 nm. Negatively charged ions or molecules such as DNA become trapped within the cathodic compartment of the membrane when the overlapping Debye layers in the nanopore disallow access of DNA to pass through the pore. The ionic strength in the cathodic compartment increases because anions in the electrolyte also experience the same type of charge exclusion as DNA. This in turn causes cations to decelerate because of the lower electric field in the region closer to the membrane in the cathodic compartment. Another factor which contributes to the increase in

local electric field is the fewer anions which pass through the membrane into the anodic compartment close to the negatively charged nanopores of PET. For membranes with permiselective nanopores, a space charge exists which is a charge depletion zone on the anodic side of the nano-channel [101] [102].

When considering that the entire system is continuous, one can describe the ion flux with the Nernst-Planck equation 3 as follows: [75]

$$\frac{\partial c_1}{\partial t} = \nabla c_i u_{hydr} - D_i \nabla c_i - \nabla^2\left(\frac{c_i z_i e D_i \varphi}{k_B T}\right) \qquad \textbf{Eq 3}$$

This equation describes the motion of chemical species is a fluid medium. The ionic concentration gradient is∇c_i , u_{hydr} is the fluidic counter flow velocity, t is time, D_i is the diffusion constant, c is the concentration of the species, z is the valence of the ionic species, e is the elementary charge, k_B is the Boltzmann constant and T is temperature, φ is the electric potential. The local electric potential and local concentration of ions can be described by the Poisson equation 4,

$$\nabla^2 \varphi = \frac{\sigma_s}{\varepsilon_o \varepsilon_r} - \frac{\sigma_f}{\varepsilon_o \varepsilon_r} = \frac{\sigma_s}{\varepsilon_o \varepsilon_r} - \frac{F}{\varepsilon_o \varepsilon_r} \sum_i z_i c_i \qquad \textbf{Eq 4}$$

where σ_f is the volume charge density of the electrolyte, σ_s is the volume fixed charge density of the membrane, F is the Faraday constant, and ε_0 and ε_r are the permittivity of the vacuum and dielectric constant, respectively.

In conclusion, it can be said that a counteracting fluid flow is required for efficient trapping and concentration of DNA in front of 100 nm pore PET membranes. The velocity of ions increases in the field amplified sample compartment and the PET membrane shows a stronger wall potential which acts on the anions resulting in more efficient trapping of DNA.

3.4. Isotachophoresis

Isotachophoresis (ITP) is derived from the Greek words *iso* which means equal, *tachos* which means speed and *phoresis* which means migration. Thus, the words liberal translation means migration at equal speed. [103]. The ITP process is a method of pre-concentration of DNA which requires a discontinuous buffer system that consists of a leading and terminating electrolyte. The fast leading electrolyte has a higher ionic concentration than the slower terminating electrolyte. As in electrophoresis, a voltage is applied which causes DNA to migrate. However, in ITP a low electric field is formed in the leading electrolyte and a higher electric field in the terminating electrolyte, causing a stacking of DNA between the two. This step of pre-concentration can be done in conjunction with capillary electrophoresis in order to couple pre-concentration and separation in a single unit. This is known as transient isotachophoresis [104]. tITP requires a separating matrix so that the DNA can stack in front of it, followed by size separation within it. In addition to the fact that ITP stacks charged molecules of the same electrophoretic mobility, it also allows for separation of molecules with different electrophoretic mobility. This method of pre-concentration was used in conjunction with electrokinetic trapping and followed by separation of DNA by capillary electrophoresis within a single device in this work.

3.5. Agarose gel electrophoresis

DNA gel electrophoresis is a method of separating DNA according to size. This is carried out by applying an electric field in order to move the negatively charged DNA molecules through the gel matrix. The term gel refers to a matrix which contains and separates DNA molecules. The gel is generally a cross-linked polymer of a certain composition and porosity based on its concentration. Agarose is however composed of long un-branched chains of carbohydrate without crosslinks, unlike the alternative; acrylamide. Separation is carried out by placing the DNA in a well within the gel and applying an electric field in such a way that the negatively charged DNA migrates towards the positively charged anode. DNA molecules separate according to the length of the DNA fragment. This phenomenon can be explained by the relation between electrophoretic mobility and size.

The Einstein relation as shown in equation 5 shows the dependence of the diffusion coefficient on electrophoretic mobility.

$$\boldsymbol{D = \frac{\mu_e k_B T}{ze}} \qquad \textbf{Eq 5}$$

In this case *z* represents the number of charged species in the ion and *e* is the elementary charge. The diffusion coefficient can be altered in the case of nucleic acids. A macromolecule such as DNA underlies a rotational (D_r) and translational diffusion coefficient (D_t) [105]. The diffusion coefficient (D_r) for a rod like molecule can be represented as follows in equation 6 [106]:

$$D_r = \frac{3k_BT\,(\ln(p)+\delta)}{\pi\eta L^3} \qquad \textbf{Eq 6}$$

p is the axial ratio *(p = L/d*) with the end-to-end length *(L)* of the nucleic acid chain, d is the diameter, the correction factor *(δ)*, thermal energy is represented by *(k_BT)* and η represents the viscosity. The translational diffusion coefficient is thus represented by the equation 7 below [106]:

$$D_t = \frac{k_BT\,(\ln(p)+\nu)}{3\pi\eta L} \qquad \textbf{Eq 7}$$

In this case, *v* is a correction for end effects and is given by v = 0.312 + 0.565/p - 0.1/p2 [105]. A measure for the end-to-end length of polymers which is sometimes consulted to model DNA polymers can be derived from the Zimm model [107] [108]. The diffusion coefficient is dependent on the electrophoretic mobility which is in turn dependent on the size of the DNA molecule. The electrophoretic mobility of double stranded DNA increases with an increase in chain length until approximately 500 bp, after which it remains constant above this length [109].

3.6. Capillary Electrophoresis

Size separation of DNA can also be carried out in long capillary tubes instead of a slab of gel. Capillary electrophoresis is designed to separate species according to their size to charge ratio within a capillary that is filled with electrolyte. The separation is also dependant on the

migration of analytes in an applied electric field. The electrophoretic migration velocity (μ_p) of an analyte toward the electrode of opposite charge is as shown in equation 8:

$$u_p = \mu_p E \qquad \textbf{Eq 8}$$

, where $\mu_p$the electrophoretic mobility and E is the electric field strength. The value of electrophoretic mobility of an analyte is directly proportional to its ionic charge and inversely proportional to frictional force that exists in the buffer. Thus, two different analytes in a sample would separate from each other should they have different charges or experience different frictional forces. Frictional forces within the buffer system are dependent on factors like viscosity (η) as well as the size and shape of the ion [110]. Thus the electrophoretic mobility of an analyte can be expressed as shown in equation 9:

$$\mu_p = \frac{z}{6\pi\eta r} \qquad \textbf{Eq 9}$$

Where z is the net charge of the analyte and r is the Stokes radius of the analyte. The Stokes radius is given by equation 10:

$$r = \frac{k_B T}{6\pi\eta D} \qquad \textbf{Eq 10}$$

Where T is the temperature and k_B is the Boltzmann constant, D is the diffusion coefficient. The equations above prove that the electrophoretic mobility, μ_p of an analyte is directly proportional to the charge of the analyte and inversely proportional to its radius. Therefore, an analyte with a higher charge would have a lower electrophoretic mobility and an analyte with

a larger radius would have a smaller electrophoretic mobility. The value of μ_p can also be determined experimentally from the migration time and field strength as shown in equation 11.

$$\boldsymbol{\mu_p} = \left(\frac{\boldsymbol{L}}{\boldsymbol{t_r}}\right)\left(\frac{\boldsymbol{L_t}}{\boldsymbol{V}}\right) \qquad \textbf{Eq 11}$$

, where L is the distance from the inlet to the detection point, t_r is the time required for the analyte to reach the detection point (migration time), V is the applied voltage (field strength), and L_t is the total length of the capillary [110]. The speed at which an analyte migrates is also dependent on electro osmotic flow (EOF) of the buffer system. Analytes migrate towards the electrode of opposite charge while being separated by differences in electrophoretic mobility. Therefore positively charged analytes migrate toward the cathode and negatively charged analytes migrate toward the anode. The speed of the electro osmotic flow, u_0 can be represented as follows

$$\boldsymbol{u_0} = \boldsymbol{\mu_o E} \qquad \textbf{Eq 12}$$

Where μ_o is the electroosmotic mobility, which is defined as:

$$\boldsymbol{\mu_0} = \frac{\boldsymbol{\epsilon\zeta}}{\boldsymbol{\eta}} \qquad \textbf{Eq 13}$$

, where ζ is the zeta potential of the capillary wall, and ϵ is the relative permittivity of the buffer solution. The speed of migration of an analyte in the electric field can be represented by the following equation:

$$\boldsymbol{u_p + u_0 = (u_p + u_0)E} \qquad \textbf{Eq 14}$$

All analytes are carried along with the buffer system towards the cathode since the electro osmotic flow of the buffer is generally greater than that of the analyte. Analytes with a larger negative charge move more slowly through the capillary column due to their conflicting electrophoretic mobility's [110]. Electro osmotic flow exists when the electric field is applied to a capillary column that contains solution as well as charges within the capillary wall. The effect of EOF can be diminished using solvents such as sodium hydroxide.

In order to calculate the resolution (R_S) of analytes such as DNA in conventional capillary electrophoresis once can use the following equation:

$$\boldsymbol{R_s = \frac{1}{4}\left(\frac{\Delta\mu_p\sqrt{N}}{\mu_p + \mu_o}\right)} \qquad \textbf{Eq 15}$$

According to this equation, resolution is best when the electrophoretic and electro osmotic mobility's are similar in magnitude and opposite in sign. It can also be seen that high resolution requires lower velocity as well as an increased analysis time. In the equation above μ is the apparent mobility in the capillary column, and N is the number of theoretical plates which can be represented as follow:

$$\boldsymbol{N = \frac{\mu V}{2D_m}} \qquad \textbf{Eq 16}$$

The number of theoretical plates represents the efficiency of separation in this case. Here, D_m is the diffusion coefficient of the analyte. Here it seems that the efficiency of separation

is limited by diffusion of analytes and is directly proportional to the electric field strength. In the case of this device, we could also determine the resolution as the level of DNA separation when exiting the capillary column. Thus a better resolution would be the seen as a larger distance separating each DNA fragment when exiting the device.

3.7. Bioanalyser

The Agilent Bioanalyser uses a type of lab on a chip technology for the analysis of DNA, RNA, proteins and even whole cells. This uses a micro-channel based electrophoretic cell that provides fast and accurate determination of these molecules. An example of this micro-channel device is shown below:

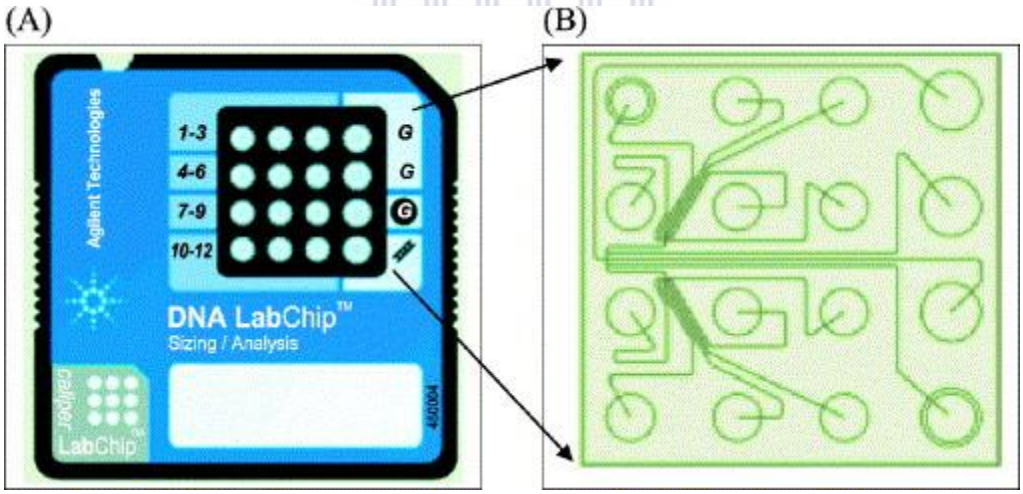


Figure 7: Agilent Bioanalyser micro-channel chip (A) external appearance of chip (B) inner architecture of chip

[111]

The chip contains 16 wells, 3 for loading the capillary gel dye mix, 1 for the molecular size ladder used as a reference and 12 for experimental samples. It also contains connected micro-channels which are filled with a sieving polymer and fluorescent dye i.e. the gel dye mix. For

the size determination of DNA fragments, the DNA will pass through these micro-channels under the influence of electrodes which create electrokinetic forces. This is very similar to the theory behind capillary electrophoresis. DNA fragments are thus intercalated with the fluorescent dye, separated by size and detected by a laser based detector within the Agilent Bioanalyser equipment. This equipment is known to have a limit of detection of down to 5 pg/µl [112].

3.8. Fluorescence Spectroscopy

Fluorescence spectroscopy is a type of spectrochemical method for analysis of molecules. During fluorescence, a molecule is irradiated by light at a certain wavelength and emits radiation of a different wavelength. This is made possible because of the fact that molecules have various states referred to as energy levels. The molecule or species is excited when a photon is absorbed and transitions from its ground state to one of the vibrational states in the excited state. Inter-molecular vibrations cause excited molecules to lose energy and drop to a lower vibrational state in the excited state. This process of excitation and loss of energy is represented by the Jablonski diagram shown below in Figure 8:

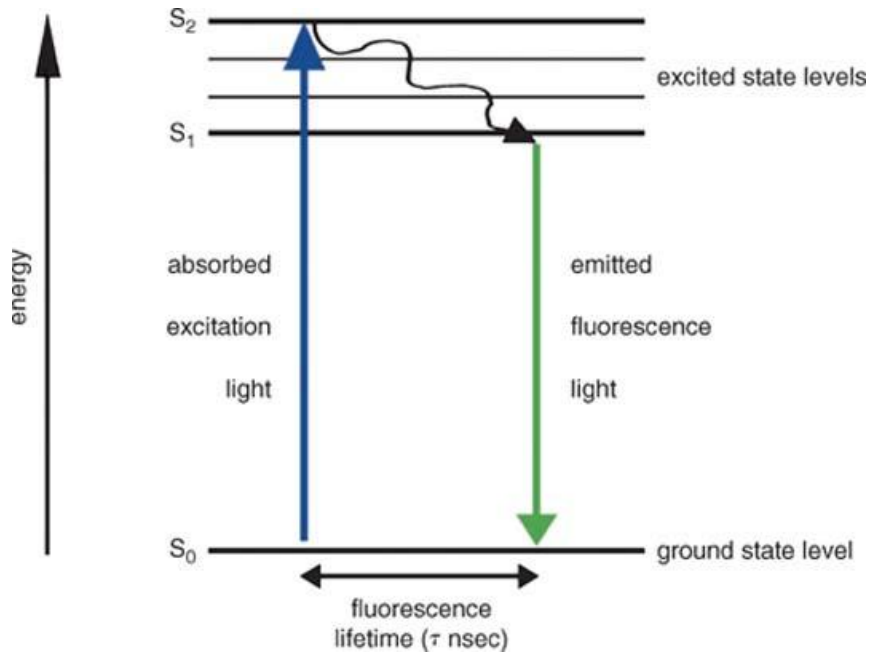


Figure 8: Jablonski Diagram representation of fluorescence

The species loses even more energy and drops back down to the ground electronic state and emits a photon in the process. This photon will have different energy and frequencies since the molecules might drop to any of the various vibrational levels. The photon emitted can be visualised as fluorescence.

Some proteins or small molecules are naturally fluorescent and others can be labelled with an intrinsic fluorophore. This fluorophore can either be a fluorescent dye, a small molecule or a quantum dot. The fluorophore used in this specific experiment is Rhodamine 6G. This molecule has an absorption/excitation wavelength of, λ_{abs} = 525 nm and an emission wavelength of, λ_{em} = 555 nm. The absorption/emission spectra as well as the structure of Rhodamine 6G is as shown in Figure 9 below:

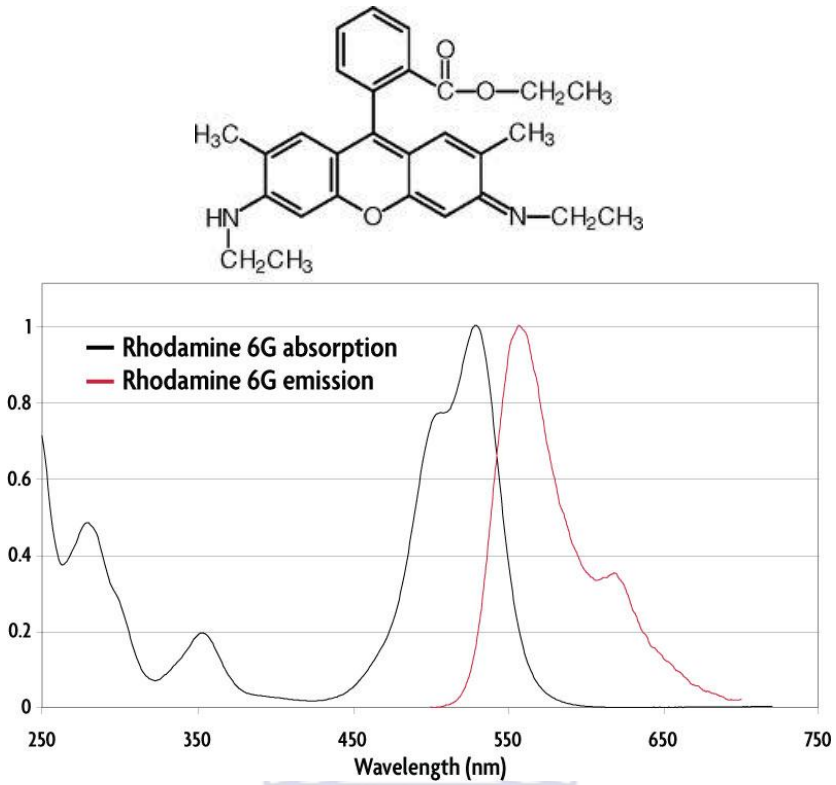


Figure 9: Absorption/excitation and emission spectra of Rhodamine 6G

Rhodamine 6G can be attached to nucleic acids and act as a label for DNA detection. Organic fluorophores such as Rhodamine 6G fluoresce due to the delocalised electrons which are able to jump a band and stabilize the energy which the molecule absorbs. Therefore most organic fluorophores are conjugated systems.

As stated before, fluorescence comes from the relaxation of a molecule from its singlet excited state to its singlet ground state. The wavelength of a molecules' fluorescence is dependent on the energy gap between these excited and ground states. The overall energy associated with fluorescence can be described below in equation 17:

$$E_{fluor} = E_{abs} - E_{vib} - E_{solv.relax} \qquad \textbf{Eq 17}$$

,where E_{fluor} is the energy of the emitted light, E_{abs} is the energy of the light absorbed by the molecule during excitation, and E_{vib} is the energy lost by the molecule from vibrational relaxation. The $E_{solv.relax}$ term comes from the necessity of a solvent cage of the molecule. This is necessary for the molecule to reorient itself in the excited state and then again when the molecule relaxes to the ground state. As shown in the equation above, the energy of fluorescence is always lower than the absorbed energy. This is why the emitted light always has a longer wavelength than the wavelength of excitation.

3.9. Real Time PCR

Real time polymerase chain reaction is a method of amplifying a specific DNA target. This is an extension of the conventional polymerase chain reaction that has existed since 1983. Dr Kary Mullis pioneered the development of this technique while working for Cetus Corporation, one of the first companies working in biotechnology. The polymerase chain reaction involves heating and cooling steps that allow amplification within a tube. The tube contains various components which allow for this amplification. These include:

- the target DNA: the DNA template that contains the sequence which is to be amplified

- free nucleotides (deoxynucleoside triphosphates, dNTP's) which are the building blocks of DNA i.e. single units of DNA bases A (Adenine), T (Thymine), G (Guanine) and C (Cytosine)

- forward and reverse primers, these are short strands of single stranded DNA which are complementary to the beginning and end of the target sequence and act as the starting point of the polymerase reaction,

- Taq polymerase, a type of enzyme that is capable of synthesising new strands of DNA. Taq polymerase is the most commonly used enzyme in PCR and RT-PCR, it originates from *Thermis Aquaticus* and is capable of acting at high temperatures

- Buffer solution, which provides a suitable chemical environment for stability of DNA polymerase as well as optimal conditions for the amplification of DNA

- Divalent cations, such as Mg^{2+} which acts as a co-factor for the DNA polymerase enzyme

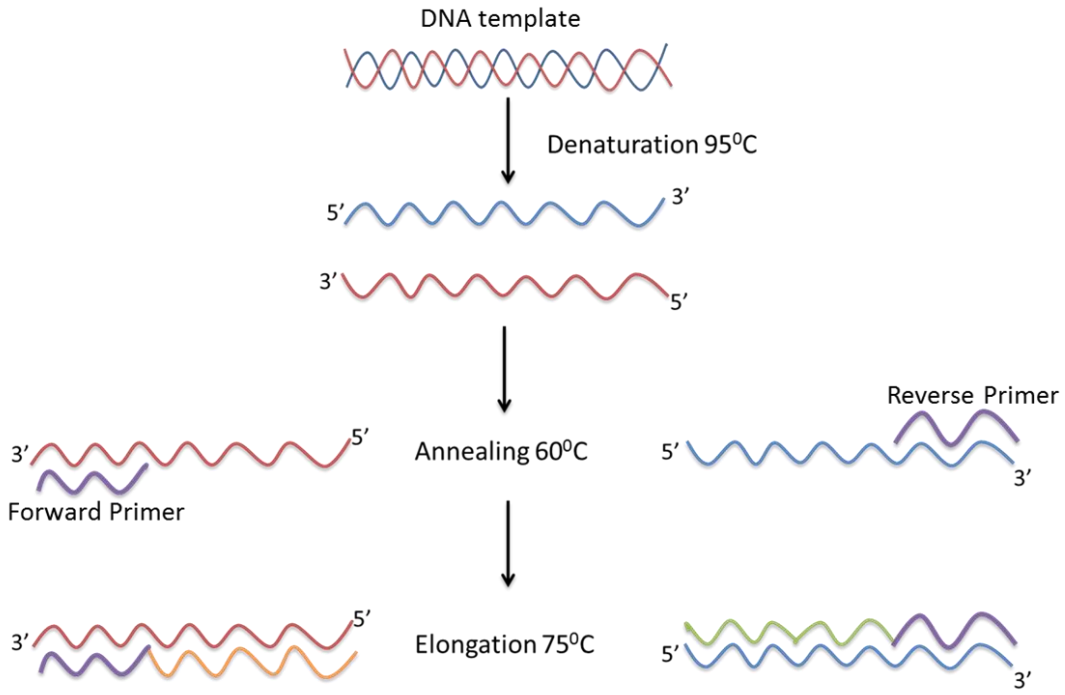
Each PCR experiment requires optimisation in each of the various components. Generally these reactions occur in small tubes of volume = 0.2-0.5 mL, but the volume of the actual reaction mixture is around 10-200 µL. These tubes are then placed in a thermo cycler that allows for the reaction to occur. PCR is basically a series of heating and cooling steps which allow for DNA amplification. Typically, these steps consist of about 20-40 cycles of heating and cooling. Each of these cycles contains three steps.

- The first step involves denaturation of the template DNA. In this case the PCR-tube is heated up to around temp = 95 ^{0}C for time = 20-30 s. At this point the double stranded DNA breaks and forms two single strands of DNA.

- The second step is annealing of the DNA primers and occurs at a temperature = 50-60 ^{0}C. The exact temperature of annealing depends on the melting temperature of the primers involved. Once the primers are annealed to the DNA template, the Taq polymerase binds to the primer-template hybrid and DNA extension can then take place

- The final step of each cycle is known as elongation and the temperature of this step is dependent on the type of DNA polymerase used. Taq polymerase has optimal activity of around temp = 75-80 ^{0}C and would thus be the elongation temperature if this polymerase enzyme is used. If another DNA polymerase is used, the temperature of the elongation step would be appropriately adjusted. The polymerase enzyme makes use of the free dNTP's in solution to extend the DNA strand by adding it to complementary DNA bases in the template DNA. The time that it takes to elongate the DNA depends on the length of the DNA template as well as the DNA polymerase enzyme.

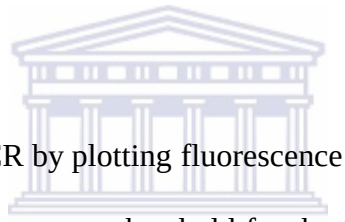
Once each cycle is complete, it is then repeated 20-40 times as stated before. After every cycle the amount of DNA present in the tube doubles and thus allows for exponential amplification of the target DNA. This amplification continues until all the reagents are used up within the PCR tube or until the DNA polymerase enzyme is loses its activity. A scheme representing conventional PCR is as shown below:



Scheme 3: Conventional PCR protocol

In comparison, real time PCR is very similar to conventional PCR explained above. It contains all the components stated above with minor modifications. Conventional PCR products are usually quantified using agarose gel electrophoresis, northern blot or southern blot, and thus require two steps i.e. amplification and then quantification. Real time PCR allows for simultaneous amplification and quantification. This is made possible by the inclusion of a fluorescent probe into the protocol with Taq polymerase as enzyme. This is a single strand of DNA that is complementary to a sequence of DNA within the template and is known as the fluorescent reporter probe. The strand of DNA is labelled with a fluorophore and quencher and produces a signal during amplification. The reaction mixture is prepared with addition of the quenched reporter probe. During the annealing stage, the reporter probe binds to the complementary sequence on the DNA template at the same time as the DNA primers. During elongation, the Taq polymerase acts in a 5′-3′ direction as an exonuclease

enzyme thereby degrading the probe. As the probe is degraded, the quencher activity is diminished and a fluorescent signal is produced. This signal is measured in real time and its increase corresponds to the amount of product and can be used to determine the threshold cycle (Ct). The threshold cycle in real time PCR is known as the amount of cycles needed to cross the background signal threshold. The Ct value is inversely proportional to the concentration of DNA target. The increase in fluorescent signal is thus directly proportional to the concentration of DNA target. Other methods of quantification in RT-PCR include the use of double stranded DNA binding dyes such as SYBR green. In this case the dye binds to all dsDNA and produces a signal which is measured after each cycle since it theoretically would bind to the amplification product.



DNA is quantified in real time PCR by plotting fluorescence versus the number of cycles on a logarithmic scale. The Ct value is set as a threshold for the fluorescent detection as a way of eliminating the background noise. In order for the protocol to be more reliable a series of dilutions of DNA is prepared to create a standard curve to monitor the change in Ct for each dilution. The slope of the linear plot is then used as a way of determining the efficiency of the RT-PCR amplification. Another difference between conventional PCR and real time PCR is the fact that RT-PCR takes place in a plate with 96 or 384 wells. Each individual well contains an individual PCR experiment that is monitored using the RT-PCR equipment.

CHAPTER 4

4.0 EXPERIMENTAL WORK I

4.1. Materials

The PMMA micro-fluidic chips were fabricated by hot embossing and the microstructures milled out at the Institut für Mikrotechnik Mainz (IMM). Acrylamide, urea, N, N dimethyl acrylamide, ammonium persulfate (APS), tris(hydroxymethyl)aminomethane (tris), N,N-dimethylurea, EDTA (ethylenediaminetetraacetic) acid and glycine were obtained from Sigma Aldrich. N,N′-methylenebisacrylamide (Bis) solution and TEMED (tetramethylethylenediamine) was purchased from Bio-Rad. PET membranes with pore size = 100 nm were purchased from Sterlitech Corporation. Agarose NA was purchased from GE Healthcare. GelStar (Lonza Group Ltd) served for staining electrophoresis gels and was purchased from Invitrogen. Hydroxypropylcellulose (HPC), hydroxyethylcellulose (HEC), polyvinylpyrrolidone (PVP) and 2-pyrrolidinone were also purchased from Sigma Aldrich. Linear polyacrylamide was purchased from GenomeLabTM.

Synthetic DNA ladder was purchased from SERVA electrophoresis GmbH. The DNA consisted of 11 fragments with the following size: 100, 150, 200, 300, 400, 500(2), 600, 700, 800, 900 and 1000 bp. A single strand of fluorescently labelled DNA was also used and purchased from Biomers.net. This DNA was 42 bp in length and was labelled with a Rhodamine 6G fluorophore with the following sequence:

5’-acc att aaa gaa aat atc att ggt gtt tcc aat ttt ttt ttt-3’

The Rhodamine 6G fluorophore was attached to the 5’ end of the DNA strand.

Real DNA samples were extracted from a maternal plasma sample of a pregnant mother carrying a male foetus. These samples obtained from the Molecular Genetics Thalassaemia Department at The Cyprus Institute of Neurology and Genetics (CING) in Nicosia, Cyprus. The DNA was suspended in a volume = 100 µL of nuclease free water and used for pre-concentration and size separation within the microfluidic device.

4.2. Buffers and solutions

All solutions were prepared in Milli Q water (Millipore Inc. Ω = 18 M Ω cm^{-1}). Leading electrolyte (LE buffer) consists of 50 mM trisHCL at pH 8.5. Terminating electrolyte (TE buffer) consists of 5 mM tris, 27.5 mM glycine at pH 8.5. 1X TBE buffer consisted of 89 mM tris, 89 mM Boric acid and 2 mM EDTA.

4.3. Instrumentation

A SynRad CO_2 (carbon dioxide) laser with a Fenix Flyer marking head was used for the fabrication of the capillary column chips. During the experiment, a high voltage sequencer HVS 448-3000D was used to carry out all the processes within the micro-fluidic device, including pre-concentration and DNA size separation. For optimisation using the Rhodamine 6G labelled DNA, a Cary 100 Bio (Varian) spectroflourimeter was used to analyse DNA

fractions at an excitation wavelength of 525 nm and emission wavelength of 555 nm. Optimisation of the protocol with the DNA ladder fragments was analysed using 2% Agarose gel electrophoresis. This type of DNA analysis was also carried out with an Agilent Bioanalyser and NanoDrop Spectrophotometer. Real Time PCR experiments were carried out using HT 7900 Life Technologies RT-PCR equipment.

4.4. Micro-fluidic device preparation

Poly(methyl methacrylate) (PMMA) chips, as shown in Figure 10 below, were cleaned by sonication in three solvents. The device was sonicated in isopropanol for 3 min in order to remove any organic matter from the micro-fluidic device, secondly in 0.5 M sodium hydroxide (NaOH) for 5 min and finally in Milli Q water for 10 min. The device was then dried under stream of nitrogen gas.

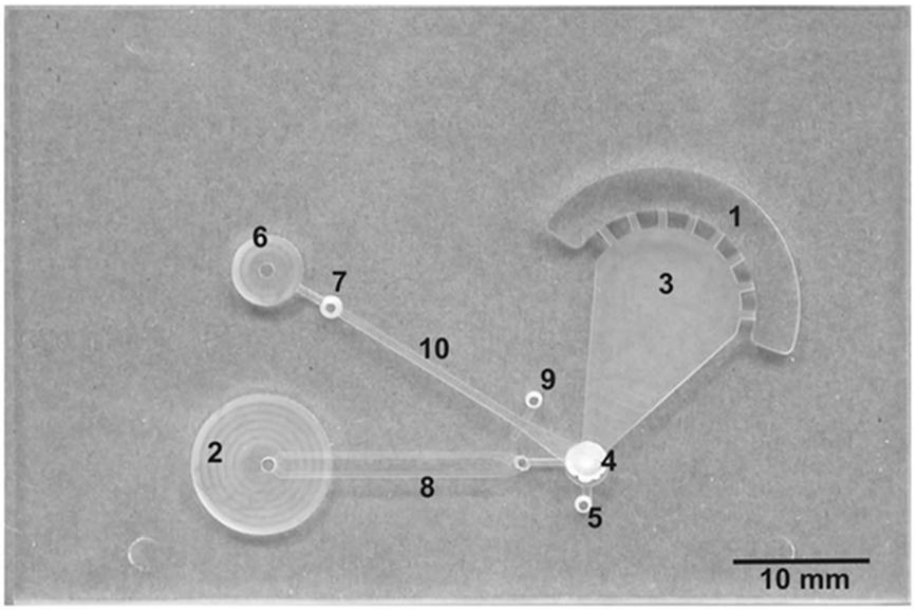
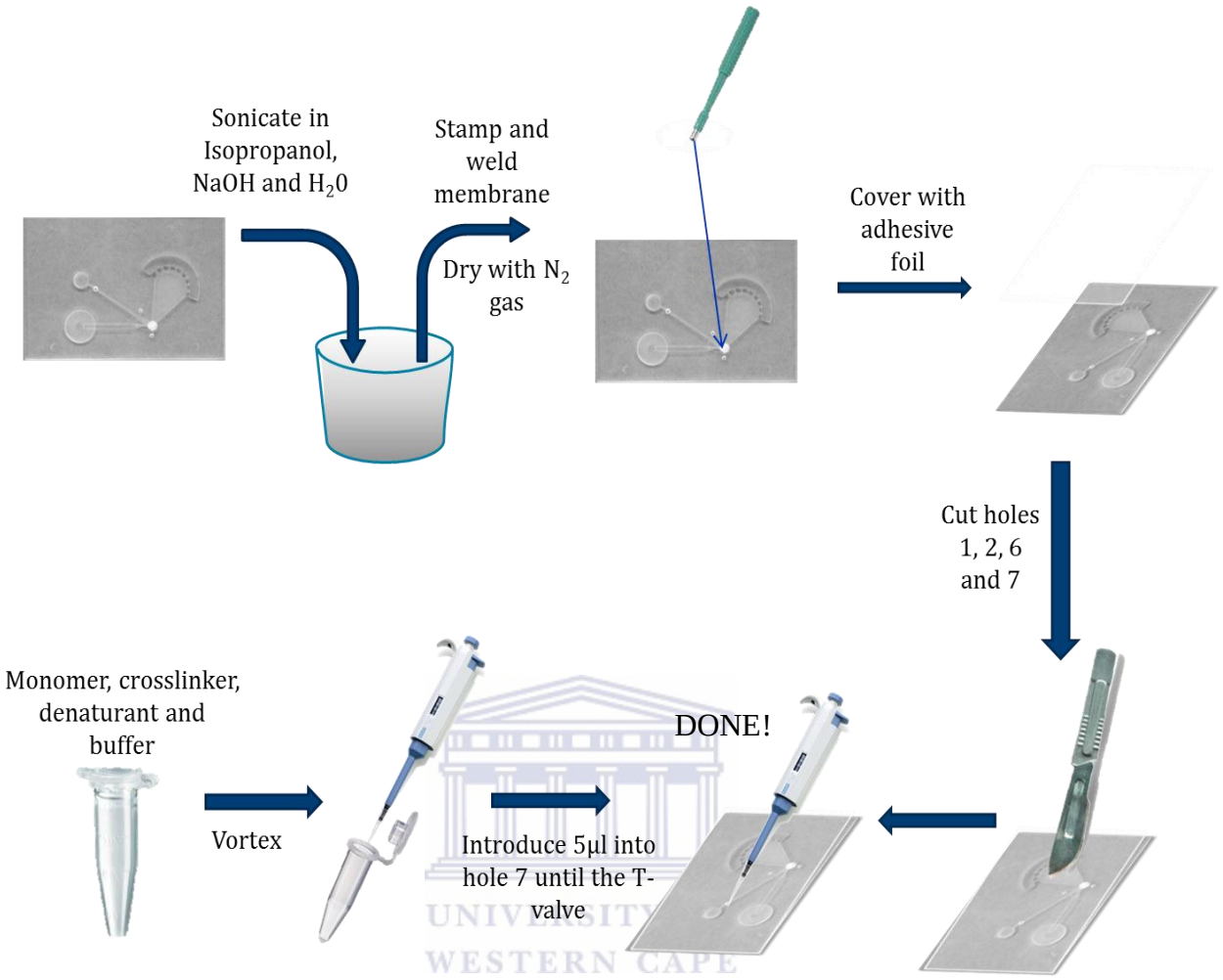


Figure 10: Design of micro-fluidic device: (1) cathode reservoir (2) anode reservoir I (3) sample reservoir, (4) membrane (5) chamber sample inlet, (6) anode reservoir II, (7) recovery outlet (8) anode channels (9) a T-crossing valve and (10) a separation channel

A 3 mm PET membrane with 100 nm pore sizes was punched and welded into the membrane chamber (4) of the device at 160 ^{0}C for 2-3 s. Thereafter both sides of the device were covered with poly(propylene) adhesive foil. Holes (1), (2), (6) and (7) were cut out to allow for addition of buffers and the matrix of the capillary column. A platform and syringe was used to flush the anode channel with TE buffer. Thereafter the separation matrix was added to the capillary column [10].

The separation matrix was prepared as follows: 9% monomer solution (acrylamide or N,N-dimethylacrylamide) with 2% Bis (N,N′-methylenebisacrylamide) and 3 M N,N-dimethylurea was dissolved in either LE buffer or 1X TBE buffer in an eppendorf tube. A 2% solution of ammonium persulfate (APS) and TEMED (tetramethylethylenediamine) was used to initiate polymerisation of the matrix; the tube was then vortexed to assist thorough mixing after which a 5 µL volume was quickly added to hole (7) until it reaches the T-crossing valve. Polymerisation can be tracked by monitoring the leftover mixture in the eppendorf tube. After the matrix has solidified, LE buffer is added to the anodic reservoir (6) as well as hole (7) to keep the matrix moist. A connection should exist between reservoir (6) and hole (7). The dilute DNA sample (synthetic or real) of 80 µL is denatured at 95 ^{0}C for 10 min and then cooled to room temperature on ice before being added to the sample chamber via hole (5). The anodic reservoir (2) and cathodic reservoir (1) is filled with TE buffer while anodic reservoir (6) is filled with LE buffer. The microfluidic device is now ready for the experiment. This process can be summarised as shown in scheme 2 below:



Scheme 4: Preparation of the micro-fluidic device

Platinum electrodes are inserted into the anodic and cathodic reservoirs and connected to a high voltage sequencer in order to apply a certain potential. Electrode A is placed in anodic reservoir (2) and A grounding is placed in cathodic reservoir (1). Electrode B is then placed in anodic reservoir (6) such that a potential gradient can exist on either sides of the capillary column. An image showing the setup of the experiment is shown in Figure 11 below:

Figure 11: Experimental Setup

The three processes within the general experimental protocol can be represented in table 2 below:

Table 2: Steps occurring during general experiment

Time (s)	Method	Voltage (V)	Comments
0-300	EKT	1000	Pre-concentration of DNA in front of PET membrane
300	-	-	Hole 9 is pierced to allow DNA transport from in front of the PET membrane to in front of the matrix in the capillary column
301-350	ITP	250/300	DNA concentrates in front of sieving matrix
351-360	Break	None	All electrodes are floating
361-end	CE	180	DNA separates in the sieving matrix/capillary column and DNA fraction are collected every 30/60seconds

The two pre-concentration steps i.e. electrokinetic trapping and isotachophoresis as well as the process of DNA separation in this case i.e. capillary electrophoresis will be explained in further detail in sections 4.6 - 4.7.

4.4.1. Column device preparation

The preparation of the column micro-fluidic device is slightly different to the protocol as explained above and will be explained separately in this section so as to avoid confusion.

Poly(methyl methacrylate) (PMMA) chips such as the one shown in Figure 12 below, were cleaned by sonication in three solvents. The device was sonicated in isopropanol for 3 min in order to remove any organic matter from the micro-fluidic device, second in 0.5 M sodium hydroxide (NaOH) for 5 min and finally in Milli Q water for 10 min. The device was then dried under stream of nitrogen gas.

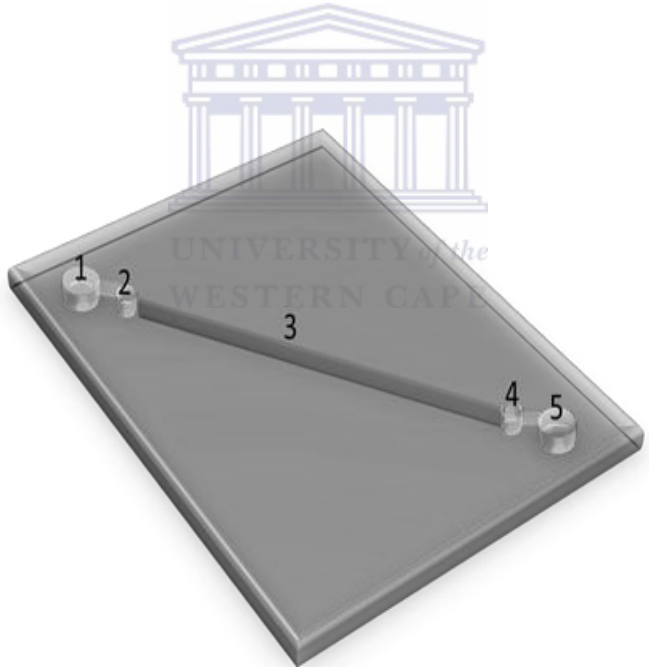


Figure 12: Design of column device: (1) Cathodic reservoir (2) Sample reservoir (3) Separation channel (4) Recovery outlet (5) Anodic reservoir

Both sides of the device were covered with poly (propylene) adhesive foil and holes (1), (2), (4) and (5) were cut out on one side using a scalpel. Thereafter the sieving matrix was prepared as follows: 9% monomer solution (acrylamide or N, N dimethylacrylamide) with 2%

Bis (N,N′-methylenebisacrylamide) and 3 M N,N-dimethylurea was dissolved in 1X TBE buffer. A 2% solution of ammonium persulfate (APS) and TEMED (tetramethylethylenediamine) was used to initiate polymerisation of the matrix; the tube was then vortexed to assist thorough mixing after which a 5 µL volume was quickly added to hole (4) until the entire capillary column was filled. Polymerisation can be tracked by monitoring the leftover mixture in the eppendorf tube. After the matrix has solidified, 1X TBE buffer is added to the anodic reservoir (5) as well as hole (4) to keep the matrix moist. A connection should exist between reservoir (5) and hole (4). The DNA sample (synthetic or real) of ±15 µL is denatured at 95 ^{0}C for 10 min and then cooled to room temperature on ice before being added to the sample reservoir (3). The cathodic reservoir (1) is filled with 1X TBE buffer and the microfluidic device is now ready for capillary electrophoresis. CE is carried out by applying a voltage of 180 V between the cathodic and anodic reservoirs for 12 min; thereafter 3 µL samples of DNA are extracted from the recovery outlet (4) at 60 s intervals and collected in eppendorf tubes for further analysis.

4.5. Electrokinetic Trapping

Electrokinetic trapping of DNA in this case is the first step in pre-concentrating the dilute DNA sample within the micro-fluidic device (Figure 10). This is carried out by applying a potential of 1000 V at electrode A in reservoir (2) for 300 s in order to concentrate the DNA in front of the PET membrane. At this point electrode A was grounded in reservoir (1) and electrode B was left floating. Thereafter, hole (9) is pierced to release the air plug formed between the membrane chamber and the matrix within the capillary column. The DNA then passes from in front of the membrane to in front of the sieving matrix in the capillary column.

4.6. Isotachophoresis

Isotachophoresis is the second step of pre-concentration. A voltage of 250/300 V is applied at electrode B in reservoir (6) for 50 s. All the other electrodes are grounded for the ITP process. This takes place in front of the sieving matrix, since an ionic gradient exists between the slower terminating electrolyte (TE) and the faster leading electrolyte (LE). This causes the DNA to stack and concentrate in front of the matrix known as a transient on column pre-concentration.

After the ITP process a ten second break occurs during which all electrodes are grounded. This allows for all the buffers in the device to be exchanged to either LE or 1X TBE buffer. This is necessary so that a homogenous buffer system exists to allow for capillary electrophoresis to take place.

4.7. Capillary electrophoresis

Capillary electrophoresis is carried out for 120 s by applying a voltage of 180 V at electrode B in reservoir 6. After which a volume of 3 µL of DNA is extracted from the recovery outlet (7) every 30 or 60 s. These 3 µL fractions are collected in eppendorf tubes and analysed either with fluorescence spectroscopy, agarose gel electrophoresis or an Agilent Bioanalyser depending on the type of DNA which is used for the experiment.

For the optimisation step of the experimental work two types of synthetic DNA was used. A 42 bp strand of DNA labelled with Rhodamine 6G fluorophores was analysed at a concentration of 90 nM. The twelve fractions collected from the micro-fluidic device was diluted in 110 µL of Milli Q water each and analysed individually. This DNA was tested using fluorescence spectroscopy at an excitation/absorption, λ_{abs} = wavelength of 525 nm and an emission wavelength, λ_{em} = 555 nm. The second type of synthetic DNA used was a 100-1000 bp DNA ladder stained with ethidium bromide. This DNA consists of 11 fragments: 100, 150, 200, 300, 400, 500 (2x), 600, 700, 800, 900 and 1000 bp. The experiment was carried out in the same way and the 12 fractions collected from the micro-fluidic device were stored in eppendorf tubes and tested initially with Agarose gel electrophoresis. A 2% gel was prepared with Agarose NA in 1X TBE buffer and stained with GelStar in order to image the gel under UV light. The DNA fractions were added to 2 µL of loading buffer and allowed to run at voltage = 150 V for 10-15 min. The gel was them imaged under UV light and the size separation of the DNA ladder in the micro-fluidic device was determined. Another method of DNA ladder analysis is the use of the Agilent Bioanalyser which is also based on gel electrophoresis but has a lower and more accurate limit of detection, as low as 5 pg/µL. This is a type of lab on a chip technology which has been described in more detail in Chapter 3 along with the other methodologies mentioned above.

CHAPTER 5

5.0. EXPERIMENTAL WORK II

5.1. Process optimisation

The micro-fluidic device consists of many components and processes which occur throughout the experiment that require optimisation. This is the bulk of the research and consists of many different components and experiments which are described in the sections below. The first set of optimisations includes the chemical make-up of the system, such as the capillary column, as well as the buffers in the different reservoirs. The original make-up of the capillary column matrix was as shown in Table 3:

Table 3: Original Column Composition

Function	Component	Optimised (yes/no)
Denaturing agent	7 M Urea	Yes
Sieving Matrix	9% Acrylamide	Yes
Buffer system	LE buffer	Yes
Crosslinking agent	0.15% Bis	No
Catalyst	0.6% TEMED	No
Radical Initiator	0.12% APS	No

The above table states the chemical aspects of the experiment which were optimised. The way in which this optimisation was carried out is explained below. Note that each optimisation experiment was repeated an average of 5 times in order to validate the results obtained.

5.1.1 Denaturing agents

Instead of 7 M urea, various concentrations of N,N-dimethylurea, tetramethylurea, 2-pyrrolidinone as well as combinations of these were tested as a denaturing agent. This was done while all other components were kept constant to have an accurate comparison.

5.1.2. Buffers

Instead of LE buffer as the continuous buffer system during capillary electrophoresis, the entire buffer system was replaced with 1X TBE (89 mM tris, 89 mM Boric Acid and 2 mM EDTA) buffer while all the other components were kept constant to have an accurate comparison. The replacement of the buffer system occurs during the 10 s break which occurs after the ITP process at second 351-360.

5.1.3. Sieving Matrix

Instead of 9% acrylamide, various concentrations of poly (N, N-dimethylacrylamide), poly (N-hydroxyethylacrylamide), hydroxyethylcellulose, hydroxypropylcellulose,

polyvinylpyrrolidone and poly (ethylene oxide) were tested as possible sieving matrices for the micro-fluidic device. These individual experiments were all carried out while keeping the other parameters constant.

Not only do the chemical aspects of this micro-fluidic device need to be optimised, but also the physical processes which occur during the experiment. These include voltage, timing as well as temperature. The optimised physical components are as shown in Table 4 below:

Table 4: Original Physical Processes

Function	Component	Optimised (yes/no)
DNA pre-concentration	EKT voltage (1000 V)	Yes
DNA pre-concentration	ITP voltage (250 V)	Yes
DNA separation	CE voltage (180 V)	Yes
Resolution of DNA separation	CE temperature (RT)	Yes
DNA separation	Extraction Time (30 s)	Yes
DNA separation	Capillary column length (1.5 cm)	Yes

5.1.4. Voltage Changes

The original voltages of the three processes are as shown in the table 4 above. The voltage of the isotachophoresis step was increased to 300 V from 250 V. This was done while keeping all other parameters of the device constant to monitor the effect on sample loss. The voltage of capillary electrophoresis was changed to 140 V, 220 V and 270 V to test the effect of DNA resolution with respect to voltage.

5.1.5. Separation Temperature

The original temperature of capillary electrophoresis was kept at room temperature of 25 ^{0}C. This temperature was increased to 40, 50 and 60 ^{0}C to monitor the effect of temperature on the separation and resolution of DNA during capillary electrophoresis while all other parameters were kept constant.

5.1.6. Extraction Times

All of the above optimisation steps were carried out using the single sized fragment of Rhodamine 6G labelled DNA, and analysed using fluorescence spectroscopy. In order to determine the resolution of DNA fragments at different extraction times, the 11 fragments of a DNA ladder (100-1000 bp) were used. The extraction times = 30 and 60 s were carried out and compared. The separated DNA fractions were collected and analysed using an Agilent Bioanalyser.

5.1.7. Column Length

The micro-fluidic device originally contains a capillary column of length = 1.5 cm. In an attempt to increase resolution or separation of the DNA ladder, a new capillary column chip was fabricated with a capillary column of length = 3 cm, double that of the original. The DNA ladder was separated in a capillary column with the optimised sieving matrix composition, and using a capillary electrophoresis setup at a voltage of 180 V for time = 12 min. The separated DNA ladder was then collected in 12 fractions at every 30 or 60 s interval; collected in eppendorf tubes and analysed using an Agilent Bioanalyser.

5.2. Application to real samples

Once the entire protocol was optimised, both the chemical and physical aspects, the micro-fluidic device were used to concentrate and separate a maternal plasma sample from a pregnant mother carrying a male foetus. The final experimental protocol can be outlined in table 5 below:

Table 5: Optimised Protocol

Component	Function	Final Value
Sieving Matrix	Acrylamide or pDMA	9%
Denaturing Agent	N, N-dimethylurea	3 M
EKT and ITP buffer	LE and TE buffer	
CE Buffer	TBE	1X
ITP	Pre-concentration of DNA	300 V
CE	DNA separation	180 V
CE Temperature	DNA resolution	RT (25 ^{0}C)
DNA extraction	DNA separation	60 s
Column Length	DNA separation	3 cm

The optimised protocol was then applied to ten real samples of DNA which contained unknown amounts of cell free foetal DNA. The 12-14 DNA fractions were collected in eppendorf tubes and analysed using Real Time PCR. The protocol is as described below:

5.2.1 Real Time PCR Protocol

Real Time PCR was used as a method to distinguish maternal DNA from foetal DNA in the maternal plasma samples. This was done by amplifying two genes: the CCR5 gene which is a general gene present in the genome of both males and females; and the DYS14 gene which is male specific. The amplification of the latter will indicate the presence of cell free foetal

DNA. The RT-PCR protocol involved the construction of a calibration plot to quantify the amount of DNA in each unknown DNA fraction. This was done by preparing increasing concentrations of male genomic DNA to plot a standard curve at concentrations of 25.4 ng/µL, 2.54 ng/µL, 0.254 ng/µL and 25.4 pg/µL. A positive control was also included in the protocol as male genomic DNA at a concentration of 175 ng/µL. A negative control in the protocol consisted of female genomic DNA which was used at a concentration of 212 ng/µL. A volume of 1 µL of the unknown fractions, the standard curve aliquots as well as the positive and negative controls were added to the wells of a 384 micro titre plate along with 4 µL of master mix for the RT-PCR amplification.

The master mix for the male specific DYS14 contained 2.5 µL TaqMan PCR mix, 0.3 µL DYS14 primer mix, 0.1 µL DYS14 probe labelled with FAM and 1.1 µL nuclease free water. The master mix for CCR5 contained 2.5 µL TaqMan PCR mix, 0.2 µL CCR5 primer mix, 0.1 µL CCR5 probe labelled with FAM and 1.2 µL nuclease free water. In the case of the positive and negative controls, as well as the standard curve master mix, the nuclease free water contained 20% 1X TBE buffer. The plate was then sealed with optical film and spun at 1000 rpm for 1 min. The real time PCR protocol was carried out by denaturing DNA for 10 min at 95 ^{0}C and then annealing for 2 min at 50 ^{0}C. Thereafter amplification was carried out by 45 cycles of 15 s at 95 ^{0}C and 1 min at 56 ^{0}C.

A diagram that represents the layout of the 384 well micro-titre plate is as shown in Table 6:

Table 6: Representation of PCR micro-titre plate layout

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	***0A_DYS***	***0A_DYS***		***0B_DYS***	***0B_DYS***		0A_CCR5			0B_CCR5			***S1_DYS***	***femgenDYS***	***femgenDYS***	***femgenDYS***		Femgen CCR5	femgen CCR5	femgen CCR5				
B	***1A_DYS***	***1A_DYS***		***1B_DYS***	***1B_DYS***		1A_CCR5			1B_CCR5			S1_CCR5	***malegenDYS***	***malegenDYS***	***malegenDYS***		malegen CCR5	malegen CCR5	malegen CCR5				
C	***2A_DYS***	***2A_DYS***		***2B_DYS***	***2B_DYS***		2A_CCR5			2B_CCR5			***S1_DYS***	***S1_DYS***	***S2_DYS***	***S2_DYS***	***S3_DYS***	***S3_DYS***	***S4_DYS***	***S4_DYS***	***Blank_DYS***	***Blank_DYS***	***Blank_DYS***	***Blank_DYS***
D	***3A_DYS***	***3A_DYS***		***3B_DYS***	***3B_DYS***		3A_CCR5			3B_CCR5			S1_CCR5	S1_CCR5	S2_CCR5	S2_CCR5	S3_CCR5	S3_CCR5	S4_CCR5	S4_CCR5	Blank_CCR5	Blank_CCR5	Blank_CCR5	Blank_CCR5
E	***4A_DYS***	***4A_DYS***		***4B_DYS***	***4B_DYS***		4A_CCR5			4B_CCR5														
F	***5A_DYS***	***5A_DYS***		***5B_DYS***	***5B_DYS***		5A_CCR5			5B_CCR5														
G	***6A_DYS***	***6A_DYS***		***6B_DYS***	***6B_DYS***		6A_CCR5			6B_CCR5														
H	***7A_DYS***	***7A_DYS***		***7B_DYS***	***7B_DYS***		7A_CCR5			7B_CCR5														
I	***8A_DYS***	***8A_DYS***		***8B_DYS***	***8B_DYS***		8A_CCR5			8B_CCR5														
J	***9A_DYS***	***9A_DYS***		***9B_DYS***	***9B_DYS***		9A_CCR5			9B_CCR5														
K	***10A_DYS***	***10A_DYS***		***10B_DYS***	***10B_DYS***		10A_CCR5			10B_CCR5														
L	***11A_DYS***	***11A_DYS***		***11B_DYS***	***11B_DYS***		11A_CCR5			11B_CCR5														
M	***12A_DYS***	***12A_DYS***		***12B_DYS***	***12B_DYS***		12A_CCR5			12B_CCR5														
N	***13A_DYS***	***13A_DYS***		***13B_DYS***	***13B_DYS***		13A_CCR5			13B_CCR5														
O	***14A_DYS***	***14A_DYS***		***14B_DYS***	***14B_DYS***		14A_CCR5			14B_CCR5														

Key for Table 5:

A/B	Repetitions of the same experimental protocol
n = 1, 2, 3 etc.	Fraction number extracted from the chip
DYS	Male specific gene to be amplified
CCR5	General gene to be amplified
S1-4	Standard concentrations of male genomic DNA to be used as a calibration curve
Femgen	Female genomic DNA (positive/negative control)
Malegen	Male genomic DNA (positive control for DYS)
Blank	Sample with no DNA present, only nuclease free water

CHAPTER 6

6.0 RESULTS AND DISCUSSION

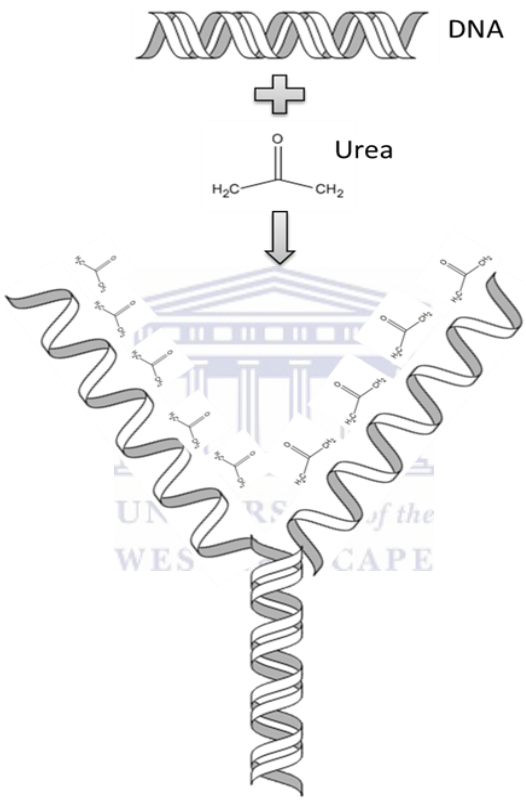
6.1. Optimisation

The optimisation of the micro-fluidic device was carried out by concentrating and separating synthetic DNA. Various parameters and processes were changed and the effect on the DNA separation and resolution was monitored using fluorescence spectroscopy, Agilent Bioanalyser and Agarose gel electrophoresis. The results of each optimisation step are discussed below:

6.1.1 Denaturing agents

DNA size separation requires both accuracy and reproducibility, but what is very important in this case, is the resolution or separation of these DNA fragments. This is because the ideal goal is to use this device to separate cell free foetal DNA from maternal DNA, by taking advantage of the size of the respective molecules. One of the components of this device which could greatly improve the resolution of DNA fragments is the addition of a denaturing agent to the capillary column matrix in which the DNA is separated. One of the most commonly used denaturing agents in electrophoresis is urea [63]. It has been shown to greatly improve the resolution of DNA fragments at high concentrations of about 7 M.

Urea denatures DNA by unfolding the secondary structure of the molecule. This forces the molecule to unfold as well as the internal hydrogen bonds of the double strand to break and produce single stranded DNA. The primary driving force of this type of denaturation is the strong interaction between urea and the bases of the DNA which have a large surface area [113]. A proposed representation of the denaturing mechanism can be represented in Scheme 5 below:



Scheme 5: Proposed mechanism of DNA denaturing by urea

In this case a single sized 42 bp fragment of Rhodamine 6G labelled DNA was added to the micro-fluidic device at a concentration of 90 nM. The fluorescence intensity of this initial DNA sample was measured using fluorescence spectroscopy and compared to each of the 12 fractions extracted from the micro-fluidic device. A representation of the experiment with 7

M urea as a denaturing agent is shown below with fluorescence intensity as a function of the fraction number in Figure 13 below:

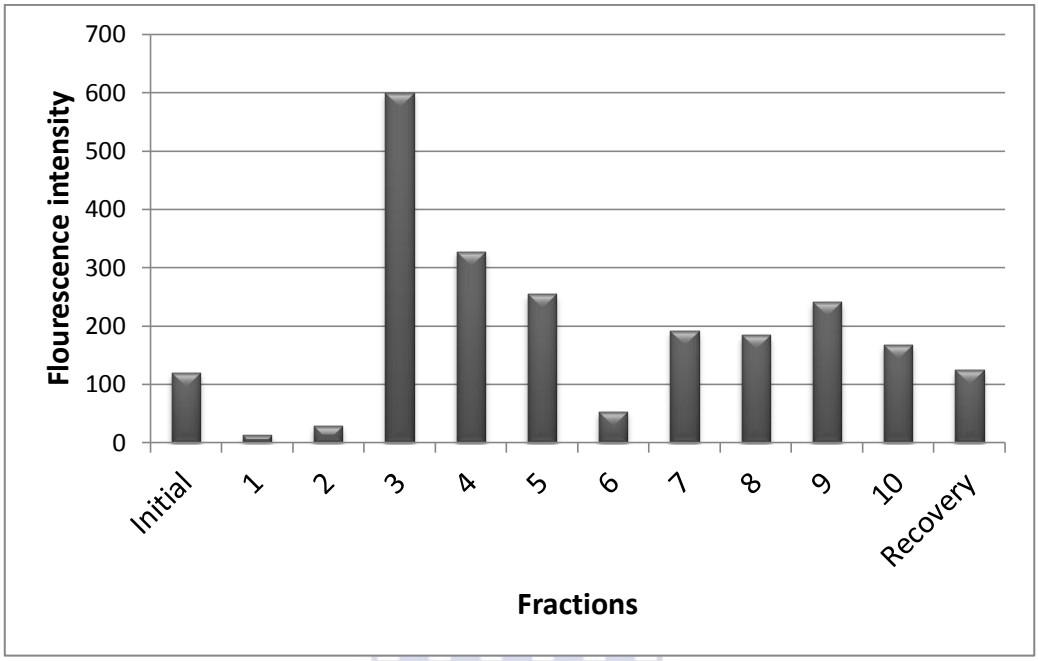


Figure 13: Separation of DNA in 9% acrylamide with 7 M urea as denaturing agent

The recovery fraction is a 10-15 µL aliquot of the liquid leftover in the sample chamber which is analysed to determine sample loss during the experimental process. Since the DNA is present only in a single sized fragment (42 bp), we expect that the DNA should exit the micro-fluidic device in a single fraction. As seen in Figure 13, the DNA does not exit in a single fraction but rather trails in all of the fractions exiting the chip. In the optimisation process one could view resolution as the ability of the single fragment to exit the chip all at the same instance. This shows that urea is a decent denaturing agent but improvements need to be made. The denaturing ability of urea has been shown to increase with an increase in alkyl substitution [59]. Therefore, together with urea, N,N-dimethylurea and tetramethylurea have been tested as a denaturant at various concentrations in the sieving matrix of the capillary column. The best results obtained from all the concentrations of denaturing agent

tested, was found to be 3 M N,N-dimethylurea. The resolution of the DNA exiting the chip with various concentrations of N,N-dimethylurea is shown below in Figure 14:

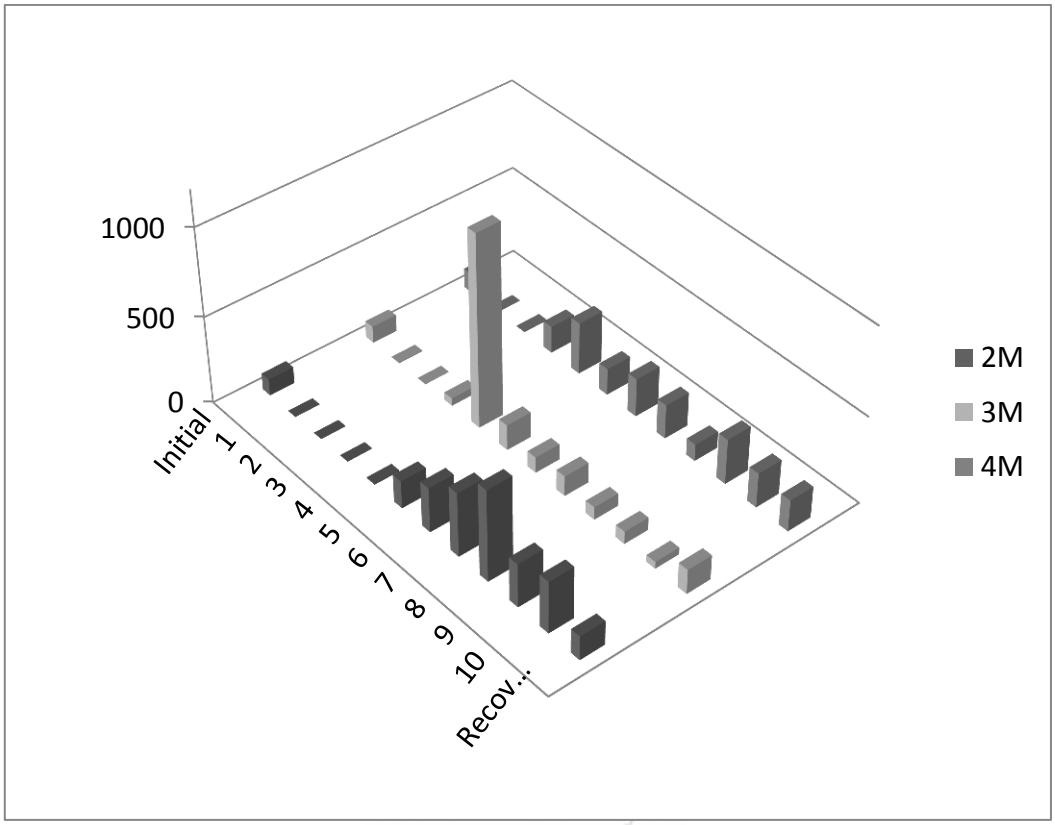


Figure 14: Separation of DNA in 9% acrylamide with various concentrations of N, N-dimethylurea

In this case one can clearly see the excellent resolution of the DNA fragment which the 3 M N,N-dimethylurea produces. In this case the DNA fragment exits the column or micro-fluidic device in a single fraction very early in the process. This is the ideal situation since small fragments of DNA should exit the device both early and in a single fraction.

Along with urea derivatives, organic solvents have also been found to improve the resolution of DNA in electrophoresis by acting as a denaturing agent. These solvents include ethanol,

methanol and pyrrolidinone. Pyrrolidinone was tested as a possible denaturant on its own as well as in combination with 3 M N,N-dimethylurea. However, it did not improve the resolution of the DNA much greater than compared to N,N-dimethylurea on its own and was thus not considered as a viable addition to the sieving matrix. From now on 3 M N,N-dimethylurea will be used as the denaturing agent in all further experiments.

6.1.2. Sieving Matrix

Acrylamide is one of the most widely used gels for electrophoresis of both proteins and DNA. In attempts to check both the versatility of the micro-fluidic device as well as other options for improving the resolution of DNA, other sieving matrices were tested. The most viable options in this case would seem to be derivatives of acrylamide such as poly(N,N-dimethylacrylamide) and poly(N-hydroxyethylacrylamide) as well as linear poly(acrylamide) (LPA). LPA has been shown to give excellent resolution of DNA fragments in terms of DNA sequencing [56] and is very widely used. However, LPA was unsuitable for the micro-fluidic device because of its high viscosity and thus difficulty in adding the matrix to the small capillary column. Since the micro-fluidic device will be dealing with biological samples such as DNA, an aqueous system is required. This requires polymers that are easily dissolvable in aqueous media. Other polymers were also considered; these include cellulose based polymers such as hydroxyethylcellulose and hydroxypropylcellulose. Cellulose based matrices are found to have a wide application in electrophoresis [58] as well as other DNA and protein separation. Polyvinylpyrrolidone has also been found to be useful as an additive to polymer matrices for DNA separation. Low viscosity, water soluble polymers such as poly(ethylene oxide) has also shown to have good resolution of DNA in capillary columns [114]. The

polymerisation of the matrix in this case has to be controllable, as well as easy for this type of micro-fluidic device. Thus out of all the polymers mentioned and tested, acrylamide and its derivatives were found to be the most viable options. Below is an image (Figure 15) representing the optimisation of the sieving matrix by comparing the resolution of a 42 bp DNA fragment labelled with Rhodamine 6G.

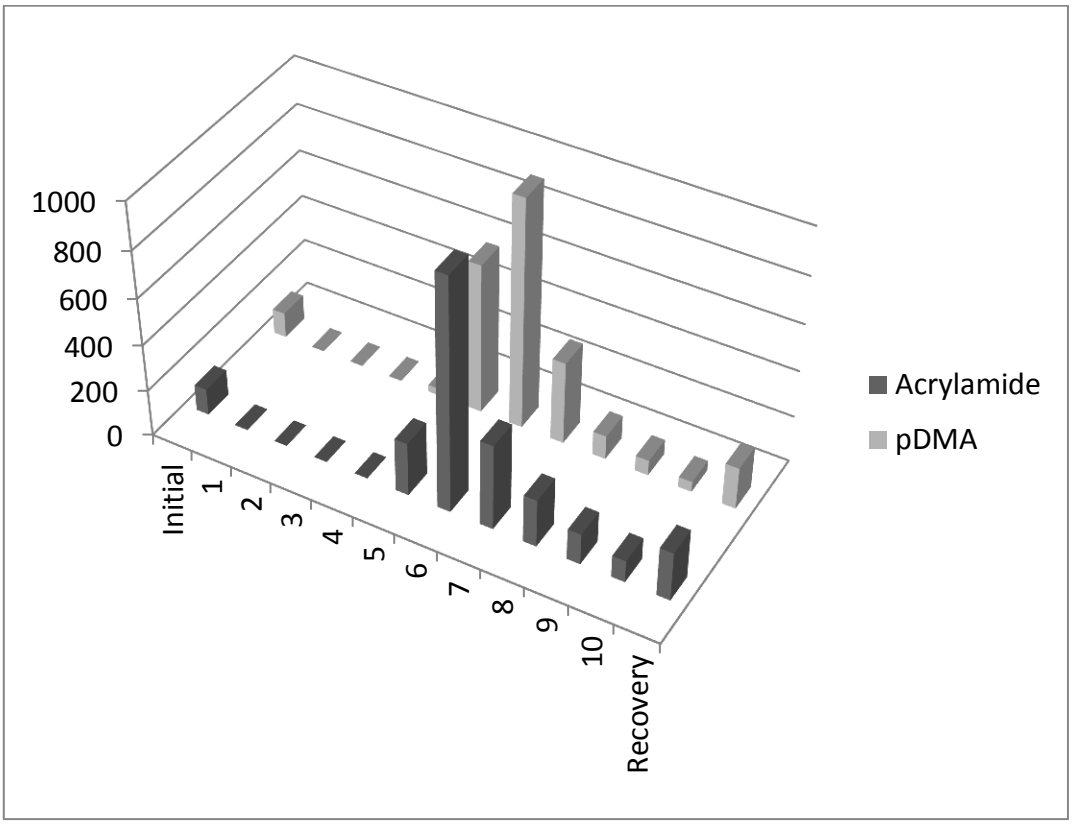


Figure 15: Acrylamide and pDMA

As can be seen in the Figure 15 above, both acrylamide as well as pDMA give excellent resolution of the single 42 bp DNA fragment. This is due to the fact that the DNA fragment exits the capillary column with the majority of DNA in a single fraction. However, tiny amounts of DNA are present on the fractions surrounding the major, which motivates us to improve DNA resolution even further.

6.1.3. Buffers

In all cases thus far, the buffers used were the conventional LE and TE buffer for EKT and ITP and only LE buffer for CE. This is also another aspect of the system which affects the resolution of the DNA fragments within the capillary matrix. 1X TBE buffer is widely used in gel electrophoresis and has been shown to have a better resolution ability compared to tris buffers which do not contain borate ions [115]. In the first two pre-concentration steps of the experimental protocol, an ion gradient has to exist for the phenomenon to occur. Thus a homogenous buffer system can only be used during CE. 1X TBE buffer was chosen to be used only during the separation process and replaced both TE and LE buffers in all the reservoirs for this step. 1X TBE buffer was found to greatly improve the resolution of the DNA fragment in this case, which can be represented by comparing a pDMA matrix in both LE and 1X TBE buffer for CE in Figure 16 below:

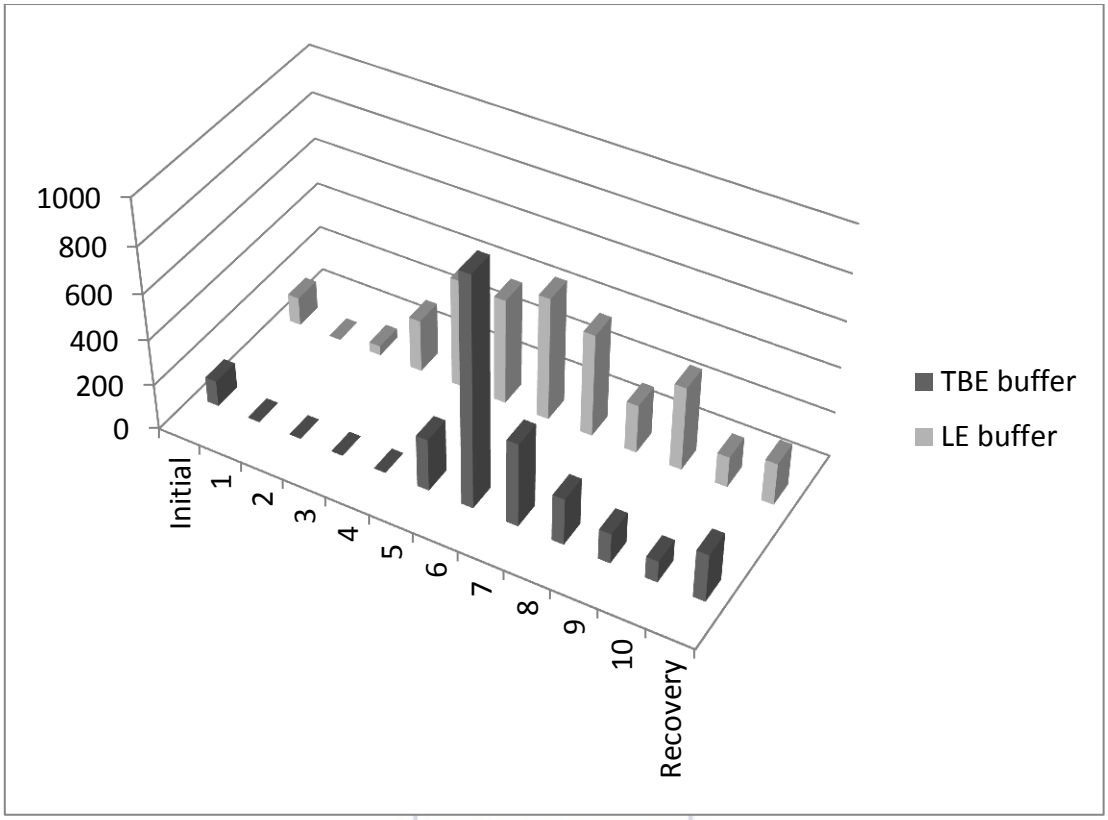


Figure 16: pDMA resolution in LE and TBE buffer for CE

pDMA as a sieving matrix in LE buffer showed a much worse resolution compared to 1X TBE buffer in this case. With LE buffer the DNA exits the column in all fractions extracted from the chip, whereas with 1X TBE buffer, the majority of the DNA exits in a single fraction. From now on, all the optimised conditions are being used in the experimental protocol, which is 9% acrylamide/pDMA, 3 M N,N-dimethylurea in 1X TBE buffer. Now that the chemical composition of the sieving matrix and buffer system is optimised, physical processes occurring within the micro-fluidic device need to be optimised as well.

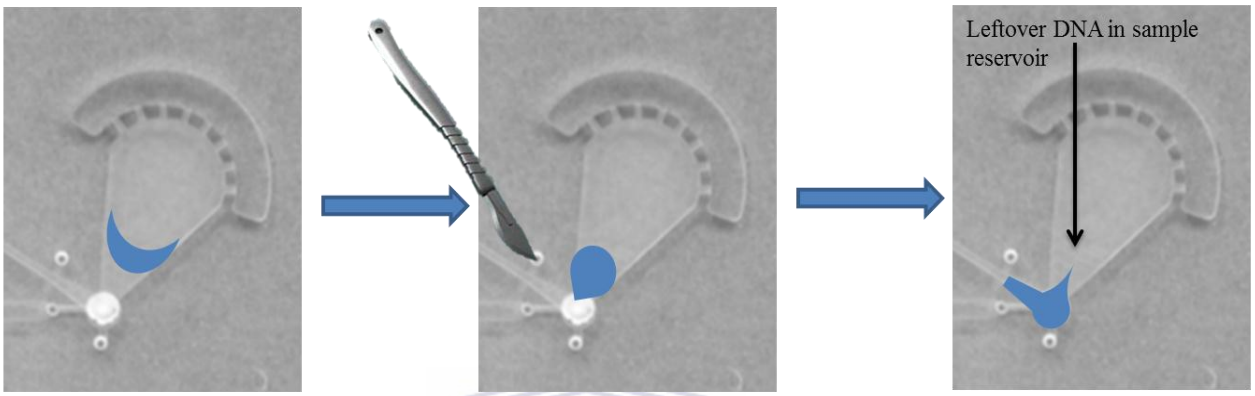
6.1.4. Separation Temperature

Many factors influence the separation of DNA, one which is important in most DNA analysis procedures, is the level of DNA denaturation. This can either be carried out using chemical denaturing agents, but also in the form of heat. DNA is known to denature or melt at temperatures higher than 90 ^{0}C by breaking the weak hydrogen bonds which hold double stranded DNA together. A denaturation temperature of 95 ^{0}C is largely used in the initial step of a PCR protocol for DNA amplification. Incorporating thermal denaturation of DNA into the capillary electrophoresis step requires a slightly lower temperature in order to maintain the structure of the gel. A temperature = 60 ^{0}C was found to be optimal for capillary electrophoresis [63] by increasing DNA resolution. However, these are usually applied to much longer and more robust capillary columns in sequencing machines. For a micro-fluidic device on this scale, gas formation becomes an issue within the micro-channels which interferes with the various processes within the device. Temperatures ranging from 40 - 60 ^{0}C were tested during capillary electrophoresis within the micro-fluidic device. The result of the applied temperature during capillary electrophoresis however was not found to greatly increase DNA resolution. Thus in this particular case it was found to be an unnecessary application or usage of energy.

6.1.5. Voltage Changes

The effect of the applied voltage of each of the steps is a vital part in controlling the migration of DNA within the device. Electrokinetic trapping has a relatively high voltage of 1000 V. However, this process has sufficiently concentrated the DNA during the optimisation process.

The second pre-concentration step of isotachophoresis has been shown to result in sample loss by inefficiently transferring the DNA from the sample chamber to stack in front of the column as shown in the scheme 6 below:



Scheme 6: Inefficient ITP process

A thin balance exists between the buffer gradient required for ITP and the time taken for the DNA to enter the sieving matrix. Thus it was not seen as a good idea to lengthen the ITP process any more than the original time = 50 s. Lengthening the time of ITP would increase the chances of the DNA entering the column instead of concentrating in front of it. Instead the voltage of this step was increased from 250 V to 300 V. A 50 V increment was chosen in order to sufficiently monitor a change within the process. Lower increments do not show a significant change in the ITP process. The migration of the 42 bp DNA fragment in this column is represented in Figure 17, by comparing the amount of DNA with an ITP voltage = 250 V and 300 V respectively.

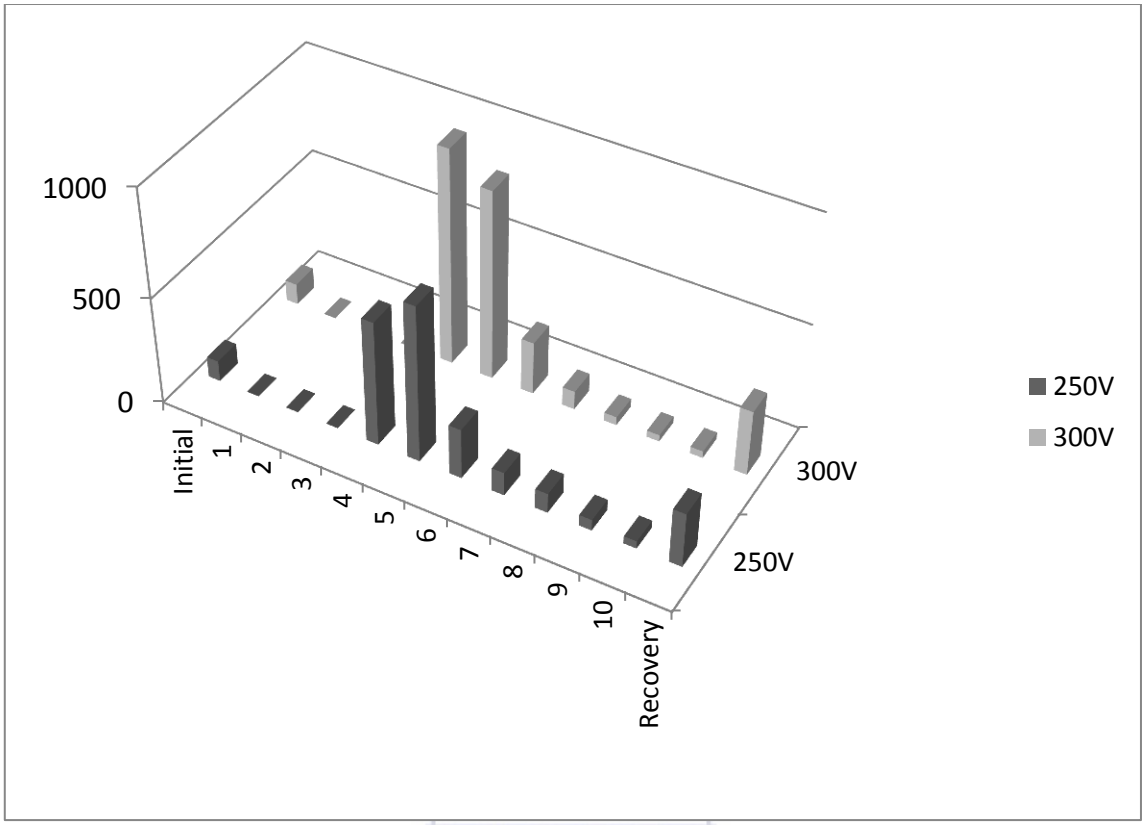
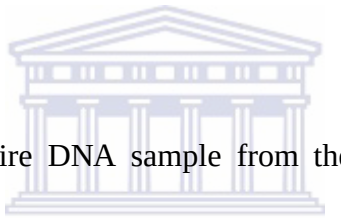


Figure 17: The effect of an increased ITP voltage on sample loss

In both these cases the same amount of DNA was inserted into the micro-fluidic device. However, from these results it is clear that a lower ITP voltage results in a lower amount of DNA which exits the micro-fluidic device. In this case there is a 30 % increase in the sample extraction with an increase of ITP voltage to 300 V. The increase of 50 V sufficiently proved that increasing the voltage decreased sample loss during ITP in this case. Therefore in all further experiments the ITP voltage was kept at 300 V. The ITP voltage can be adjusted further in future experiments with various concentrations of DNA, synthetic or real.

Now that the DNA loss is minimised within the device, we can try and have optimal separation and also resolution of the DNA fragments. This can be done using the same

rationale as before and thus changing the voltage of capillary electrophoresis. The original value of voltage = 180 V showed good resolution with the acrylamide matrix, however with the pDMA matrix the migration of DNA was slightly slower. In attempts to increase the speed with which the DNA migrates through the capillary column, higher voltages were tested. Capillary electrophoresis voltages of 220 V and 270 V with the pDMA matrix were tested i.e. at increments of 40 V to ensure an accurate comparison and differentiation. These were found to increase the speed with which the DNA migrates through the column, but have a negative effect on the resolution of the DNA fragment. Previously it was possible to extract the 42 bp DNA fragment in a single fraction, whereas with the higher voltages the DNA was extracted in about 2 fractions. Thus the voltage = 180 V for the pDMA matrix was retained.



In an attempt to capture the entire DNA sample from the acrylamide matrix in a single fraction, a lower voltage of capillary electrophoresis was also tested i.e. at 40 V lower than the original: voltage = 140 V. One would think that the DNA would migrate slower through the matrix and thereby have a better resolution by allowing a longer time for the DNA to separate. However, the migration was much slower and resulted in very bad resolution. In this case one can assume that the applied voltage was not sufficient enough to promote efficient capillary electrophoresis. Therefore in all cases from now on, a general voltage of 180 V was used during capillary electrophoresis. The next parameter which might assist in the resolution of DNA might be the timing at which the DNA is extracted from the micro-fluidic device.

6.1.6. Extraction Times

To truly compare the effect of the timing of extraction on DNA resolution and size separation, more than one fragment of DNA is required. In this case a 100-1000 bp synthetic DNA ladder was used. The optimised protocol using a 9% acrylamide matrix, 3 M N,N-dimethylurea in 1X TBE buffer for CE was used to pre-concentrate and separate this DNA ladder in the micro-fluidic device. Here extraction times = 30 and 60 s were compared. This type of DNA was initially analysed using Agarose gel electrophoresis. In cases where the concentrations of the fragments were too low for visualisation on an agarose gel, an Agilent Bioanalyser was used. This instrument is based on the same theory as gel electrophoresis but has a better limit of detection. The Agilent Bioanalyser electrophoresis gel results representing the 30 s extraction time is as shown in Figure 18 below:

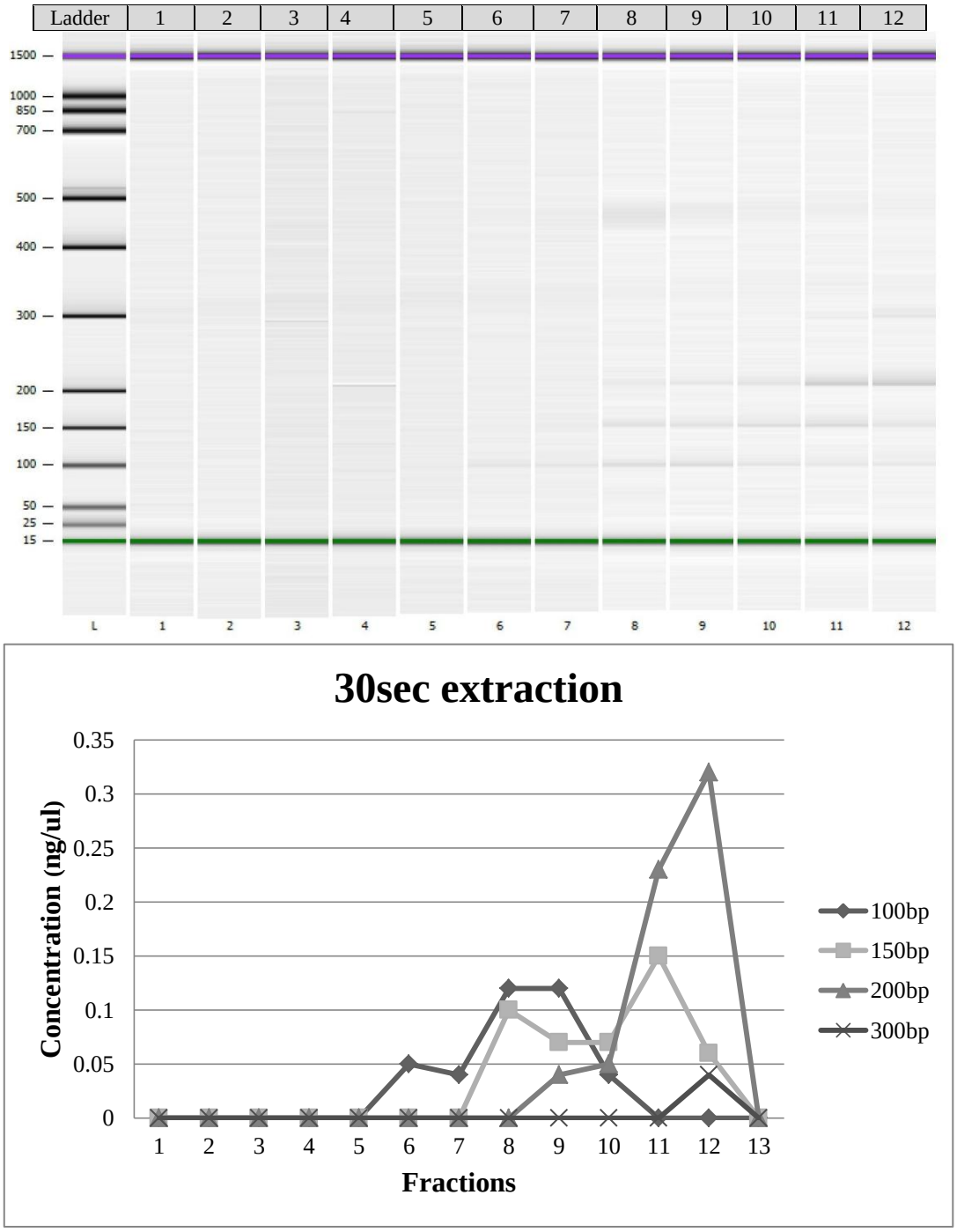


Figure 18: (a) Gel image of DNA ladder separation with 30 s extraction (b) Representation of DNA concentration in each fraction extraction at 30 s intervals

In this case, the EKT process is carried out for 300 s, ITP for 50 s and CE for 120 s. Thereafter a 3 µL sample is extracted from the micro-fluidic device every 30 s. The graph above represents the gel image in terms of the concentration of each DNA fragment in the ladder which is present in each fraction extracted from the micro-fluidic device. As we can see, the smaller sized fragments exit the micro-fluidic device earlier in the protocol compared to the larger fragments. This proves that the experiment works in terms of separating DNA according to size. However, the resolution of this experiment can be improved further. Currently, it is possible to separate the 100 bp fragment in fraction 6. However fraction 7 contains fragments of 100 bp and 150 bp together. These results are good but the concentrations of each of these fragments are still low. We went on to test the separation of the DNA ladder with 60 s extractions. The results are as shown in Figure 19 below:

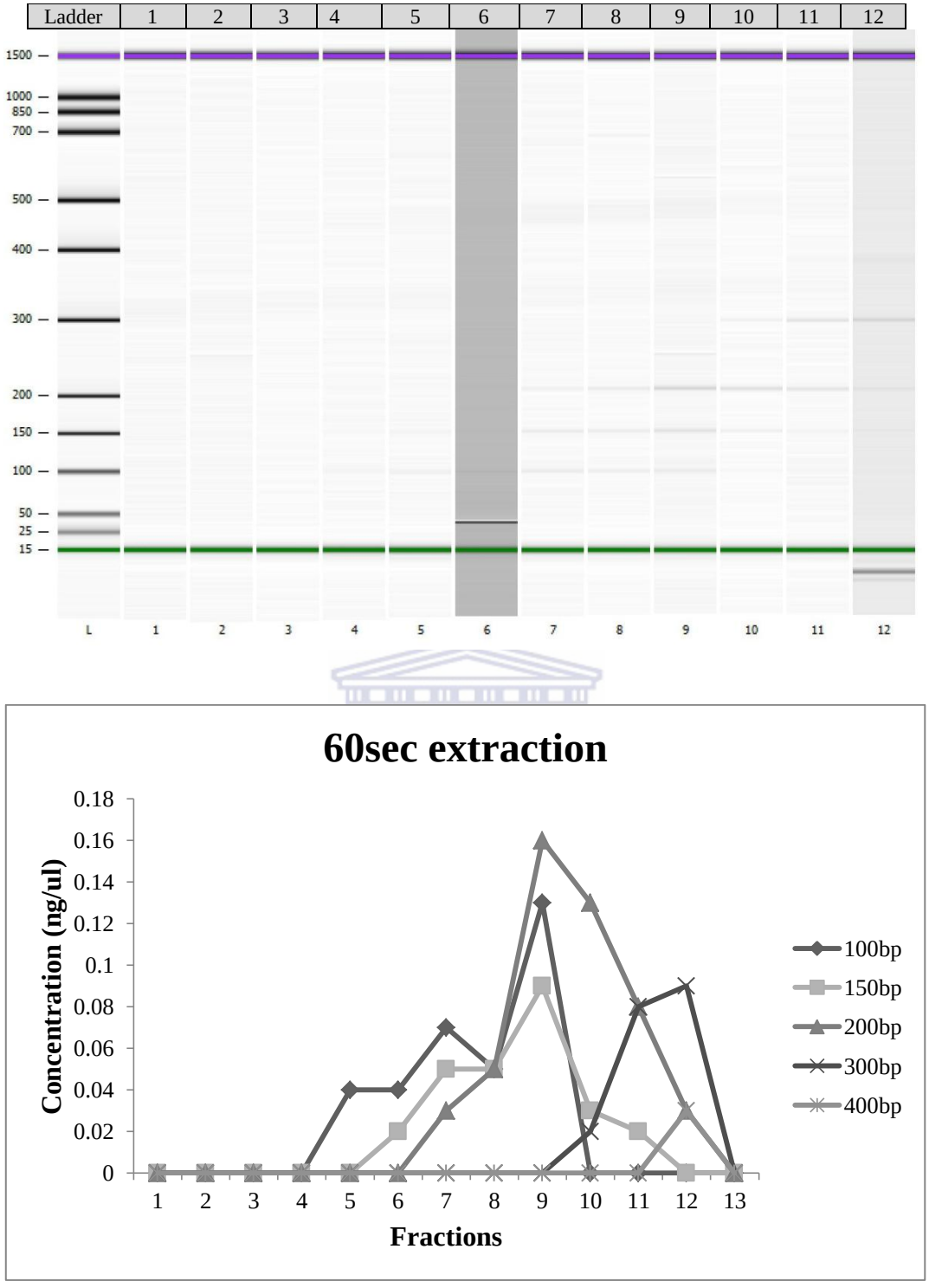


Figure 19: (a) Gel image of DNA ladder separation with 60 s extraction (b) Representation of DNA concentration in each fraction extraction at 60 s intervals

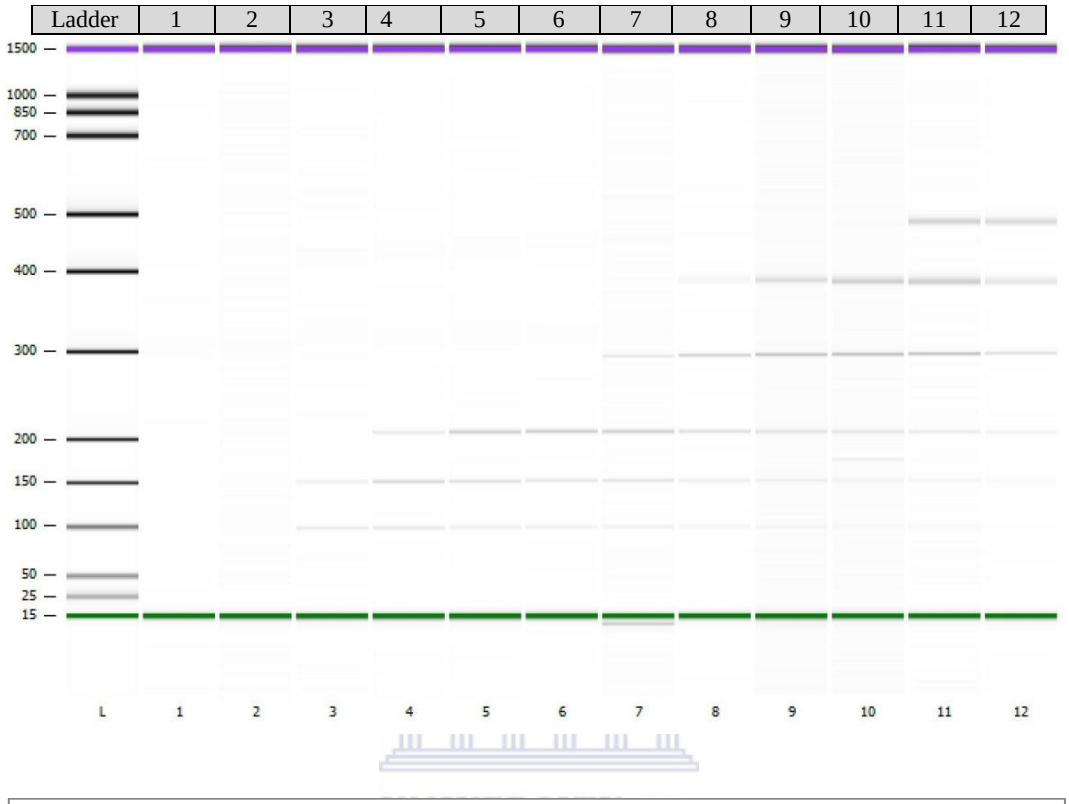
As we can see from these results, the resolution of DNA is basically the same as with the 30 s extraction time. The only difference in this case is that the DNA has more time to collect in

the recovery outlet. Here the same trend occurs, with the 100 bp fragment being isolated in an earlier fraction, and then present along with the 150 bp fragment in the next fraction. We thus concluded that the time = 60 s for extraction was better. The longer extraction time did not interfere with the resolution and also allowed the DNA to collect in the recovery outlet. This is an added benefit when dealing with real DNA samples, since more DNA can be used for further analysis. We went on to test further parameters which could possibly affect the resolution/separation of the DNA ladder within the microfluidic device.

6.1.7. Column Length

So far the device contained a capillary column which was fixed at 15 mm in length. It is proposed that an increase in this capillary column length could in fact increase the resolution of the DNA fragments by allowing more time and space in which the fragments could separate. Previous work has shown that this theory is valid i.e. an increase in column length has increased resolution of DNA fragments [116]. A new micro-fluidic device was fabricated which contained only a capillary column with double the length as that of the previous one. This was the maximum length of column achievable on the PMMA substrate using the CO_2 laser. Longer columns could be achieved if they were produced in different designs instead of just a straight line. However, this design was chosen so as to achieve a maximum consistency between the parameters and to allow for accurate comparisons. In this case, the DNA ladder concentration added to the sample reservoir was optimised in such a way that the experiment is comparable to those carried out previously. Capillary electrophoresis in a 9% pDMA matrix was carried out and fractions of DNA were extracted at time = 60 s intervals from the

recovery outlet of the micro-fluidic device. The results of the Agilent Bioanalyser analysis are as shown below in Figure 20:



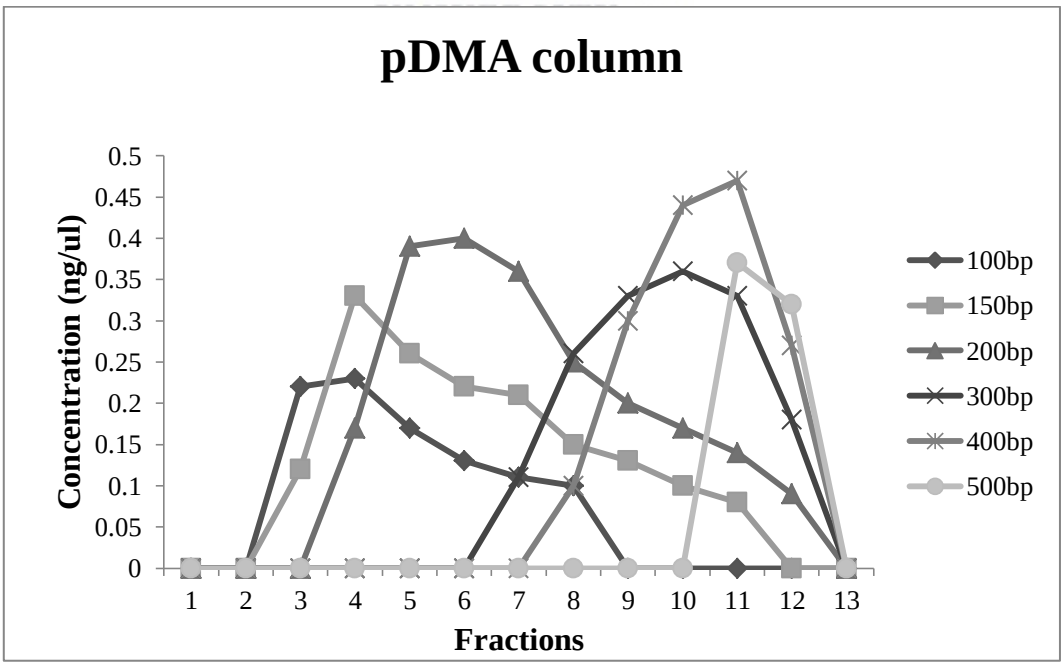


Figure 20: (a) Gel image of DNA ladder separation in 30 mm capillary column (b) Representation of DNA concentration in each fraction extracted from capillary column

As we can see, a lot more DNA is present within the protocol timing. This is much more favourable compared to previous experiments. Not only is there more DNA present in each fraction, the separation of the individually sized DNA fragments are greatly improved. Although each fragment is not isolated completely in a single fraction, each size has a maximum concentration (C_{max}) in one of them as shown in Table 7 below:

Table 7: Relative maximum concentrations of DNA fragments exiting the microfluidic device

Fragment	C_{max}	Fraction
100 bp	0.23 ng/µL	4
150 bp	0.26 ng/µL	4
200 bp	0.36 ng/µL	5
300 bp	0.36 ng/µL	10
400 bp	0.47 ng/µL	11
500 bp	0.37 ng/µL	11

Here we can see that it is possible to collect and isolate larger concentrations of the smaller fragments of DNA. All fragments that are smaller than 300 bp can be completely isolated from the rest in the first 6 fractions extracted from the device. This is a benefit if the micro-fluidic device will be used as a method of separating cell free foetal DNA from a maternal plasma sample. This is because cffDNA exists in the mother's blood stream in fragments less than 300 bp in length. Thus, in future the device will be re-designed in order to incorporate capillary columns that are 30 mm or even longer in order to separate DNA fragments with excellent resolution. Although these complete devices were not manufactured for use in this

work, the theory that a longer capillary column gives better resolution of DNA, has been successfully proven.

6.2. Application to Real Samples

Now that all the parameters within the device have been successfully optimised and incorporated into a final experiment, it will be applied to real sample analysis. As stated before, these samples were obtained from women pregnant with male foetuses. DNA was isolated from the blood of the mother and used in these experiments. The protocol can be summarised again as follows:

The matrix which was inserted into the capillary column contained either 9% acrylamide/pDMA as a sieving matrix, 3 M N,N-dimethylurea as a denaturing agent. Once the sieving matrix was fixed, 10X 80 µL real DNA samples were analysed separately, they were each added to the sample reservoir through hole 5 of the device. Buffers used for EKT and ITP processes were the conventional LE and TE buffers. All buffers were replaced with 1X TBE buffer for capillary electrophoresis. EKT voltage remained at 1000 V for 300 s. ITP was carried out at voltage = 300 V for 50 s and CE at voltage = 180 V. Extraction of DNA from the device was carried out at 60 s intervals starting at minute 8 until 14 fractions were collected. Each of these fractions were analysed using Real Time PCR. The protocol involved the amplification of two genes: DYS14 which is male specific and CCR5 which is present in both males and females. In this way it is made possible to distinguish whether each fraction contains male or female DNA. If the DYS14 gene is amplified, the DNA belongs to the foetus

and is considered cell free foetal DNA. If the CCR5 gene is amplified and NOT the DYS14 gene, we can assume that this DNA belongs to the mother. Keeping in mind that cell free foetal DNA is usually less than 300 bp in length, this DNA should theoretically be present in the initial samples extracted from the chip, since smaller fragments of DNA has been proven to move through the matrix quicker. Therefore, the larger maternal DNA should be present in the later fractions, since this DNA is usually more than 500 bp in length and thus move more slowly through the sieving matrix. For the purpose of clarity, only one RT-PCR experiment is represented here.

As proof that the RT-PCR protocol is successful, the standard curve of each gene i.e. DYS14 and CCR5 is shown below. These standard curves also indicate the presence of the positive controls, which is male genomic DNA for the DYS14 gene and female genomic DNA for the CCR5 gene. It also shows that the negative controls were not amplified i.e. female genomic DNA with DYS14 amplification. The protocol representing the amplification of the CCR5 gene is shown in Figure 21 below:

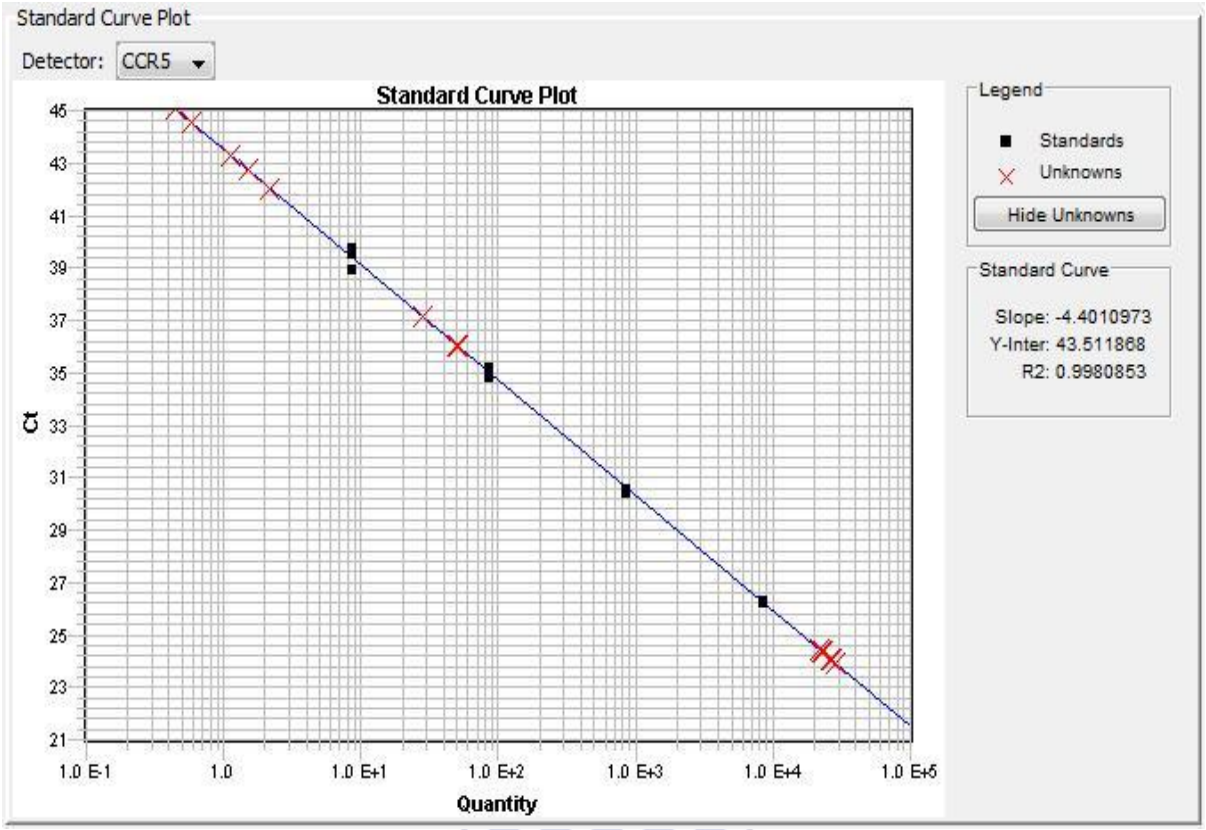


Figure 21: Standard Curve representation of CCR5 amplification

According to The Cyprus Institute of Neurology and Genetics (CING), who provided the RT-PCR protocol, the slope of the standard curve has to be in the range of -3.5 to -4.5. Therefore we can conclude that this experiment was successful since the slope is -4.4 as well as the fact that the standards were reproducible. The positive controls for CCR5 gene contained female genomic DNA at a concentration of 212 ng/µL. According to the RT-PCR results, the positive controls contained an average of 191 ng/µL with a standard deviation of 4,38 ng/µL. A graph which represents the amplification of these positive control samples are as shown in Figure 22 below.

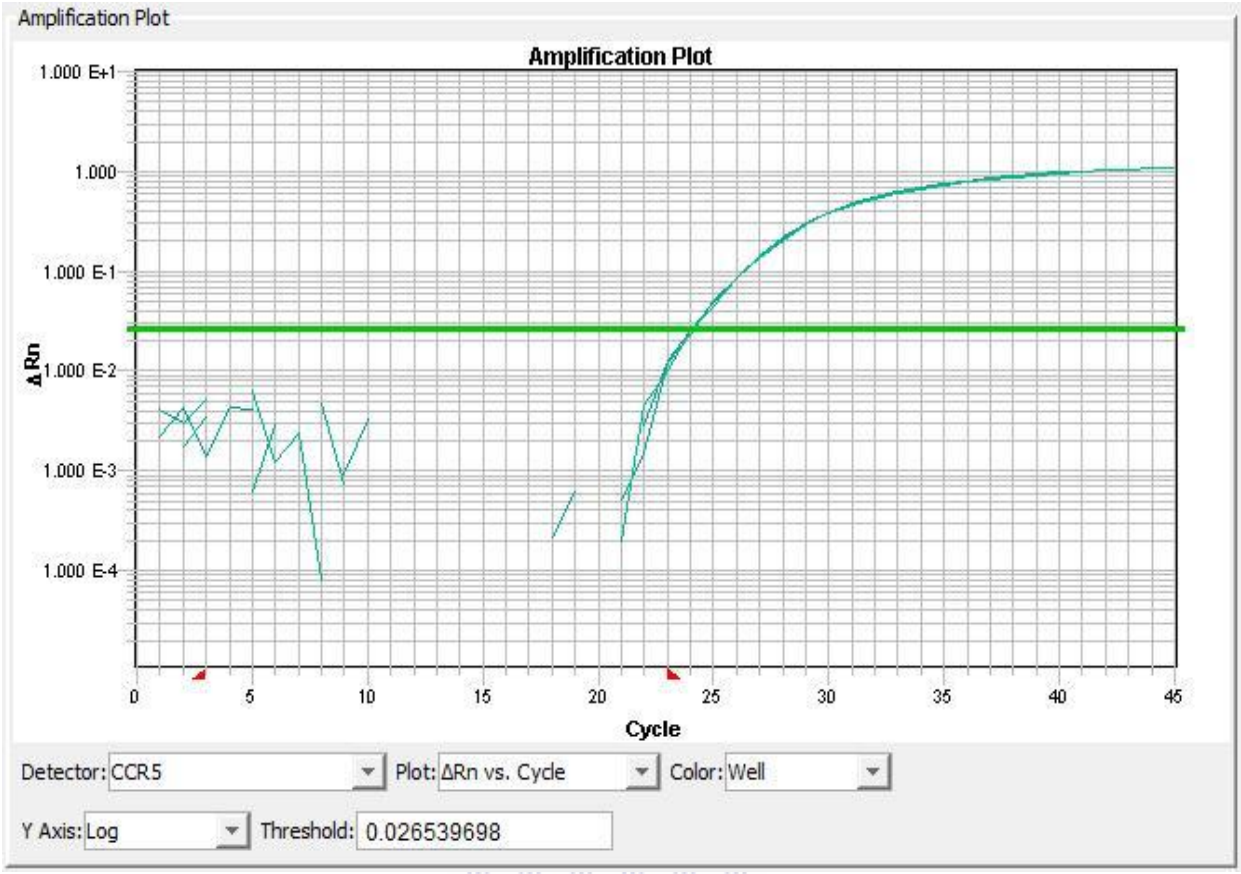


Figure 22: Amplification of CCR5 gene in positive control samples

This shows that the protocol gives a result that is in the range of ±21 ng/µL accuracy for the positive controls.

The theoretical initial concentration of cell free foetal DNA was calculated to be 4.46 pg/µL since this is the amount of DNA amplified by the DYS14 gene. One can conclude that the total amount of DNA that the initial samples contained would be those amplified by the CCR5 gene. This amount was calculated to be 319 pg/µL, making the cell free foetal DNA approximately 2% of the total maternal plasma DNA sample in this case. The graph which represents the DYS14 amplification is as shown in Figure 23:

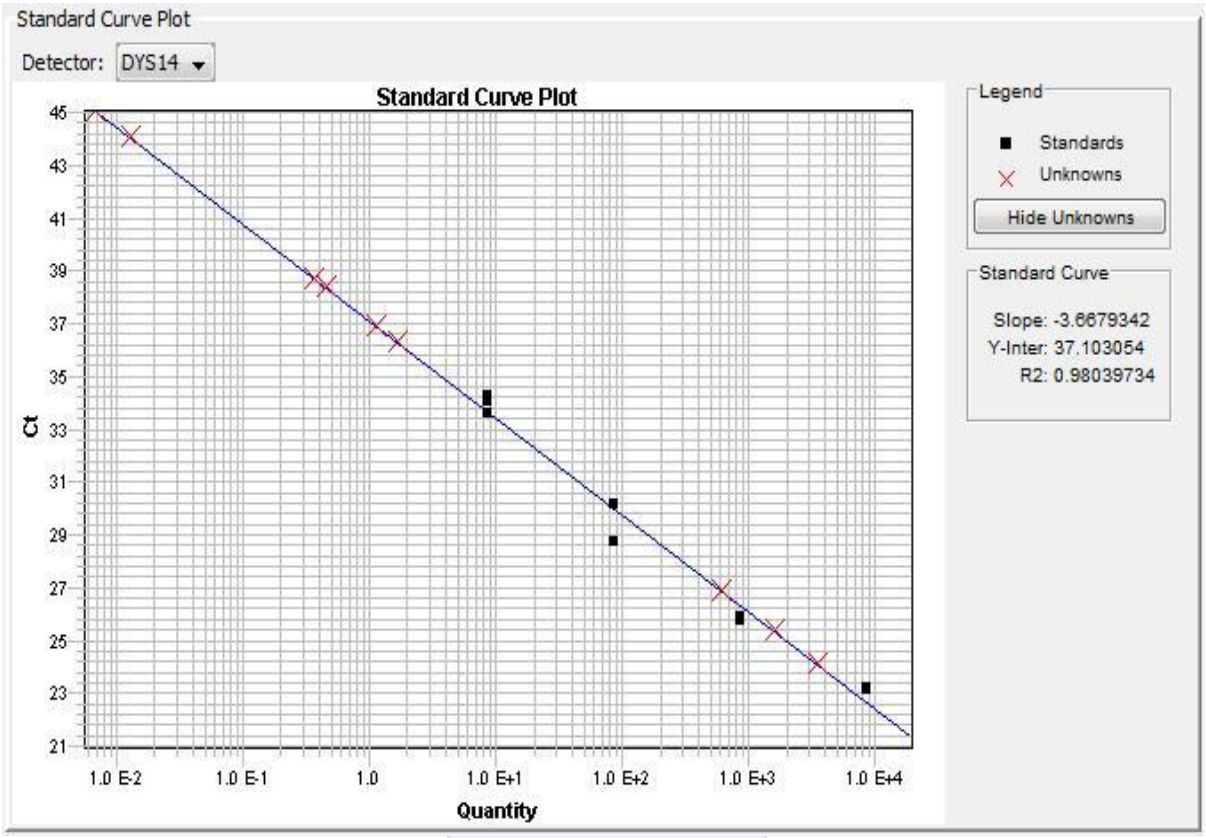


Figure 23 Standard Curve representation of DYS14 amplification

In the standard curve representing the DYS14 gene protocol, the slope is -3.638. This is within the required range of -3.5 to -4.5 and can be considered confirmation that the experiment was successful for the DYS14 amplification. The positive controls contain a high concentration of male genomic DNA (175 ng/µL) and is thus at the far right of the graph which show a high quantity of DNA and also a low Ct value. The Ct values can be defined as the number of cycles that are required for the fluorescent signal to cross the threshold or background signal and are inversely proportional to the concentration of the target DNA. This means that a lower Ct value indicates a higher concentration of target DNA and that this DNA had to undergo less amplification cycles before it was detectable by the equipment. The

results of the real time PCR can also be represented by a graph which shows the amplification of each gene by plotting the Ct value versus fraction number in Figure 24 below:

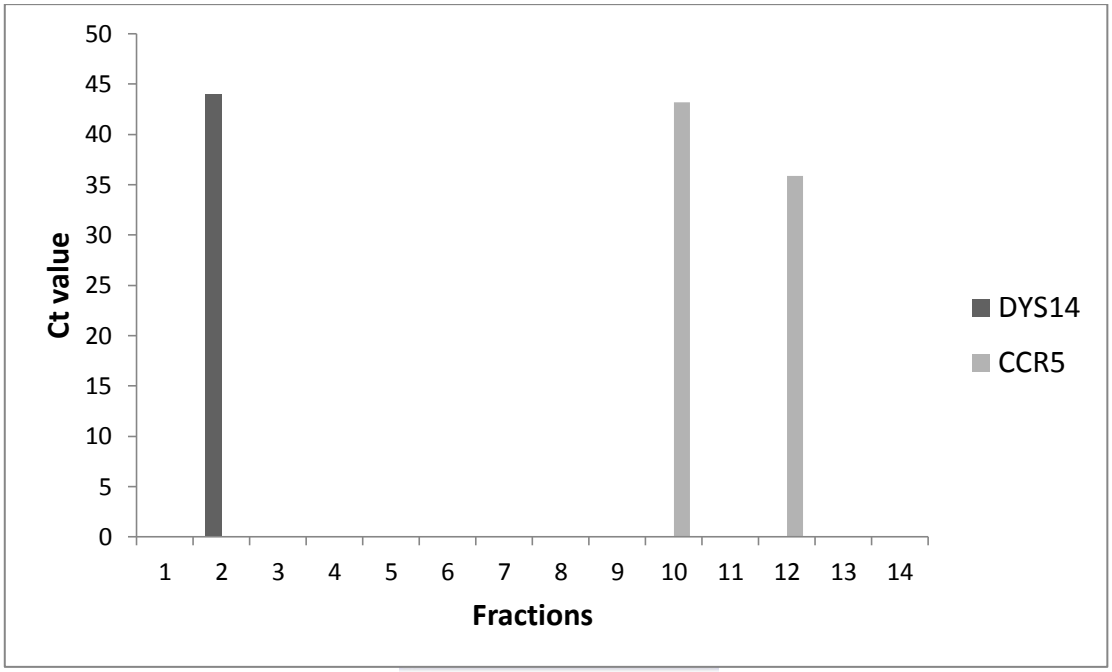


Figure 24: Representation of the presence of cell free foetal DNA in initial samples extracted from micro-fluidic device

The initial DNA samples were also represented on the standard curve plot, with an average concentration of 4.46 pg/µL as stated previously. The second fraction which exited the chip shows amplification of the DYS14 gene, with a concentration of 0.04 pg/µL and a Ct value of 43.99. The 10^{th} and 12^{th} fraction exiting the chip contained the CCR5 gene since this gene was amplified. The fact that only CCR5 was amplified in these fractions, as well as the fact that they occur so late in the process, confirms that this DNA belongs to the mother. This is because maternal DNA is larger in size compared to foetal and obviously does not contain the DYS14 gene. The concentration of DNA in fraction 10 was calculated to be 7.14 pg/µL of

DNA with a Ct value of 43.2. The DNA present in fraction 12 had a concentration of 317,24 pg/µL and a Ct value of 35.9.

Since the micro-fluidic device separated DNA according to size, we can assume that this DNA fragments were in the range of 100-1000 bp. This is because the device was optimised using synthetic DNA in this range. During the protocol only fragments up to 500 bp were collected within the time frame shown here. Fragments that are greater in size than 500 bp can possibly be collected if the protocol time was extended. Maternal DNA exists in larger fragments than 500 bp and thus not all of the DNA that exists in the sample might be represented in the graph. Leftover DNA will probably still be present either in the sample chamber or capillary column of the micro-fluidic device.

The fact that this concentration is far less concentrated than the initial sample shows that one of the pre-concentration steps within the micro-fluidic device had failed. We are positive that all processes occurred in the optimisation protocol, and can thus conclude that problems occurred because of the nature of the real samples and its effect on the EKT step. It was noted that the current flow during EKT when dealing with real samples was considerably lower compared to the synthetic DNA. We can thus attribute the decrease in DNA concentrations present in the fractions to an inefficient EKT process. Although the experimental protocol was carried out on all ten real samples, the results of only one of them is shown here. This is due to the fact that there were several issues which arose when dealing with real sample analysis which resulted in irreproducible outcomes. The trend of results however should remain the same with all ten real samples. This problem will have to be resolved in future with a more extensive study concerning real sample analysis with the micro-fluidic device.

Nonetheless, we can conclude that the micro-fluidic device successfully isolated cell free foetal DNA from at least one maternal blood sample, since the DYS14 gene was amplified in an initial fraction, separate from the maternal DNA which was present in later fractions.

CHAPTER 7

7.0. CONCLUSION

A non-invasive prenatal test was proposed by taking advantage of the presence of cell free foetal DNA in the maternal blood stream. In this work, a microfluidic device was designed and optimised for the separation of cell free foetal DNA from maternal blood to allow for further genetic testing. Cell free foetal DNA is characteristically short in length, i.e. less than 300 bp, and can thus be separated by size from larger DNA. Here it is carried out by using advances in micro-fluidics and the development of a device capable of separating DNA according to size. The micro-fluidic device used three processes in order to isolate and separate cell free foetal DNA. All the components and processes occurring within the device were optimised and tested using synthetic DNA. The optimised protocol used a 9% acrylamide/pDMA sieving matrix with 3 M N,N-dimethylurea as a denaturing agent in which to separate the DNA. For electrophoresis a homogenous buffer system of 1X TBE was found to improve DNA separation and thus resolution at a voltage of 180 V and extraction time of 60 s. The isotachophoresis worked best at a voltage of 300 V and resulted in 30% reduction in sample loss. The optimised protocol was then applied to real sample analysis obtained from women pregnant with male foetuses. The micro-fluidic device successfully isolated cell free foetal DNA from a maternal plasma samples. This was proven to be accurate using real time PCR analysis and amplification of a sex related gene.

7.1 Recommendations

In future, I would recommend that the device incorporate longer capillary columns in which to separate the DNA in order to have a better resolution and size separation. Also the micro-fluidic device should be altered and further optimised in order to appropriately analyse real samples. The design of the device can also be altered in order to enhance each process occurring. Further studies can be done to test whether the device can be re-used for more than one experiment.

REFERENCES

[1] C. Waits, B. Morgan , R. Kastantin and R. Ghodssi, "Microfabrication of 3D silicon MEMS structures using gray-scale lithography and deep reactive ion etching," *Sensors and Actuators A: Physical,* vol. 119, pp. 245-253, 2005.

[2] R. Jena, S. Chester, V. Srivastava, C. Yue, L. Anand and Y. Lam, "Large-strain thermo-mechanical behavior of cyclic olefin copolymers: Application to hot embossing and thermal bonding for the fabrication of microfluidic devices," *Sensors and Actuators B: Chemical,* vol. 155, pp. 93-105, 2011.

[3] A. Di Gaspare, L. Mariucci, A. Pecora and G. Fortunato, "Stable p-channel polysilicon thin film transistors fabricated by laser doping technique," *Thin solid films,* vol. 487, pp. 232-236, 2005.

[4] C.-C. Liu, "Development of chemical sensors using microfabrication and micromachining techniques," *Materials Chemistry and Physics,* vol. 42, pp. 87-90, 1995.

[5] G. Moore, "Cramming more components into integrated circuits," *Electronics,* vol. 38, no. 8, April 1965.

[6] I. Kobayashi, S. Hirose, T. Katoh, Y. Zhang, K. Uemura and M. Nakajima, "High aspect ratio through-hole array microfabricated in a PMMA plate for monodisperse emulsion production," *Microsystem Technology,* vol. 14, pp. 1349-1357, 2008.

[7] C. McDonald and G. Whitesides, "Poly(dimethylsiloxane) as a material for fabricating microfluidic devices," *Accounts of Chemical Research,* vol. 35, no. 7, pp. 491-499, 2002.

[8] Y. Liu, D. Ganser, A. Scheider, R. Liu, P. Grodzinski and N. Kroutchinina, "Microfabricated polycarbonate CE devices for DNA analysis," *Analytical Chemistry,* vol. 73, no. 17, pp. 4196-4201, 2001.

[9] J. Voldman, M. Gray and M. Schmidt, "Microfabrication in Biology and Medicine," *Annual Review of Biomedical Engineering,* vol. 1, pp. 401-425, 1999.

[10] Y. Sera, N. Matsubaru, K. Otsuka and S. Terabe, "Sweeping on a microchip: concentration profiles of the focused zone in micellar electrokinetic chromotography," *Electrophoresis,* vol. 22, pp. 3509-3513, 2001.

[11] Z. Long, Z. Shen, D. Wu, J. Qin and B. Lin, "Integrated multilayer microfluidic device with a nanoporous membrane interconnect for online coupling of solid-phase extraction to microchip electrophoresis," *Lab Chip,* vol. 7, pp. 1819-1824, 2007.

[12] J. Li , L. Huang, L. Ye, Z. Zhou, G. Xiang and L. Xu, "Determination of imatinib mesylate and related compounds by field amplified sample stacking with large volume sample injection capillary electrophoresis," *Journal of Pharmaceutical and Biomedical Analysis,* vol. Article in press, 2012.

[13] K. Hoeman, J. Lange, G. Roman , D. Higgins and C. Culbertson , "Electrokinetic trapping using a titan nanoporous membranes fabricated using sol-gel chemistry on microfluidic devices," *Electrophoresis,* vol. 30, pp. 3160-3167, 2009.

[14] D. Liu, M. Shi, H. Huang, Z. Long, X. Zhou, J. Qin and B. Lin, "Isotachophoresis preconcentration integrated microfluidic chip for highly sensitive genotyping of the hepatitis B virus," *Journal of Chromatography B,* vol. 844, pp. 32-38, 2006a.

[15] K. Sunberg , J. Bang, S. Smidt-Jensen, V. Brocks, C. Lundsteen, J. Parner, N. Keiding and J. Philip , "Randomised study of risk of foetal loss related to early amniocentesis versus chorionic villus sampling," *Lancet,* vol. 350, no. 9079, p. 6970703, 1997.

[16] D. Smith, "First-Trimester Nuchal Translucency Screening to Detect Down Syndrome," *Newborn and infant nursing reviews,* vol. 8, no. 1, pp. e1-e6, 2008.

[17] D. Lo, N. Corbetta, P. Chamberlain, V. Rai, I. Sargent, C. Redman and J. Wainscoat, "Presence of fetal DNA in maternal plasma and serum," *The Lancet,* vol. 350, pp. 485-487, 1997.

[18] P. Vigil-De Garcia, "Pregnancy complicated by pre-eclampsia-eclampsia with HELLP syndrome," *International Journal of Gynecology & Obstetrics,* vol. 72, pp. 17-23, 2001.

[19] S. Phelps, R. Fisher, A. Partington and E. Dykes, "Prenatal ultrasound diagnosis of gastrointestinal malformations," *Journal of pediatric surgery,* vol. 32, no. 3, pp. 438-440, 1997.

[20] B. Braden, A. Ignee, M. Hocke, R. Palmer and C. Dietrich, "Diagnostic value and clinical utility of contrast enhanced ultrasound in intestinal diseases," *Digestive and liver disease,* vol. 42, pp. 667-674, 2010.

[21] V. Scotet, I. Duqueperoux, M. Audrezet, S. Audebert-Bellanger, M. Muller, M. Blavau and C. Ferec, "Focus on cystic fibrosis and other disorders evidenced in fetuses with sonographic finding of echogenic bowel: 16-year report from Brittany, France," *American Journal of Obstetrics and Gynecology,* vol. 203, no. 6, pp. 592.e1-592.e6, 2010.

[22] M.-L. Chen, C.-H. Chang, C.-H. Yu, Y. Cheng and F.-M. Chang, "Prenatal diagnosis of cleft palate by three-dimensional ultrasound," *Ultrasound in medicine and biology,* vol. 27, no. 8, pp. 1017-1023, 2001.

[23] B. Benacerraf, V. Barss and L. Laboda, "A sonographic sign for the detection in the second trimester of the fetus with Down's syndrome," *American Journal of Obstetrics and Gynecology,* vol. 151, pp. 1078-1079, 1985.

[24] T. Kuroda, Y. Kitano, T. Honna, H. Sago, S. Havashi and M. Saeki, "Prenatal diagnosis and management of abdominal diseases in pediatric surgery," *Journal of padiatric surgery,* vol. 39, no. 12, pp. 1819-1822, 2004.

[25] (CEMET), "Randomised trial to assess safety and fetal outcome of early and midtrimester amnioncentesis," *Lancet,* vol. 351, no. 9098, pp. 242-247, 1998.

[26] F. Vandenbossche, J. Horovitz, F. Guyon, C. Verret and R. Saura, “Pain experience during chorionic villus sampling and amniocentesis: A preliminary study,” *European journal of obstretics and Gynecology,* vol. 136, pp. 189-193, 2008.

[27] Z. Appelman, C. Vinkler and B. Caspi, “Chorionic villus sampling in multiple pregnancy,” *European journal of obstretics and gynecology,* vol. 85, pp. 97-99, 1999.

[28] F. Duchatel, J. Oury, B. Mennesson and J. Muray, “Complications of diagnostic ultrasound-guided percutaneous umbilical blood sampling: analysis of a series of 341 cases and review of the literature,” *European Journal of Obstetrics & Gynecology and Reproductive Biology,* vol. 52, no. 2, pp. 95-104, 1993.

[29] R. Quintero, W. Morales, C. Kalter, M. Allen, G. Mendoza, J. Angel and R. Romero, “Transabdominal intra-amniotic endoscopic assessment of previable premature rupture of membranes,” *American Journal of Obstetrics and Gynecology,* vol. 179, no. 1, pp. 71-78, 1988.

[30] E. Kontopoulos, M. Gualtieri and R. Quintero, “Successful in utero treatment of an oral teratoma via operative fetoscopy: case report and review of the literature,” *American Journal of Obstetrics and Gynecology,* vol. 207, no. 1, pp. e12-e15, 2012.

[31] S. Illanes, M. Denbow, C. Kailasam, K. Finning and P. Soothill, “Early detection of cell-free fetal DNA in maternal plasma,” *Early Human Development,* vol. 83, pp. 563-566, 2007.

[32] D. Lo, M. Tein, T. Lau, C. Haines, T. Leung, P. Poon, J. Wainscoat, P. Johnson, A. Chang and M. Hjelm, “Quantitative analysis of fetal DNA in maternal plasma and serum: implications for non-invasive prenatal diagnosis,” *American Journal of Human Genetics,* vol. 62, pp. 768-775, 1998.

[33] M. Alberry, D. Maddocks, M. Jones, M. Abdel Hadi, S. Abdel-Fattah, N. Avent and P. Soothill , “Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast,” *Prenatal Diagnosis,* vol. 27, pp. 415-418, 2007.

[34] Y. Lo, K. Chan, E. Chen, P. Jiang, Y. Zheng, T. Leung, T. Lau, C. Cantor and R. Chiu, "Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus," *Clinical Chemistry,* vol. 54, pp. 1664-1672, 2008.

[35] S. Kim, J. Lim, S. Park, M. Kim, J. Choi and H. Ryu, "Non-invasive prenatal determination of fetal gender using QF-PCR analysis of cell-free fetal DNA in maternal plasma," *Clinica Chimica Acta,* vol. 413, pp. 600-604, 2012.

[36] R. Rijnders, G. Christiaens, B. Bossers, J. van der Smaqt, c. van der Schoot and M. de Haas, "Clinical applications of cell-free fetal DNA from maternal plasma," *The American College of Obstetricians and Gynecologists,* vol. 103, no. 1, pp. 157-164, 2004.

[37] P. Larrabee, K. Johnson, E. Pestova, M. Lucas, K. Wilber, E. LeShane, U. Tantravahi, J. Cowan and D. Bianchi, "Microarray Analysis of Cell-Free Fetal DNA in Amniotic Fluid: a Prenatal," *The American Journal of Human Genetics,* vol. 7, pp. 485-491, 2004.

[38] A. Farina, E. Leshane, G. Lambert-Messerlian, J. Canick, T. Lee, G. Palomaki and D. Bianchi, "Evaluation of Cell-free Fetal DNA as a Second-Trimester Maternal Serum Marker of Down Syndrome Pregnancy," *Clinical Chemistry,* vol. 49, no. 2, pp. 239-242, 2003.

[39] A. Bustamante-Aragones, M. Trujillo-Tiebas, J. Gallego-Merlo, M. Rodriguez de Alba, C. Gonzales-Gonzales, D. Cantalapiedra, C. Avuso and C. Ramos, "Prenatal diagnosis of Huntington disease in maternal plasma: direct and indirect study," *European Journal of Neurology,* vol. 15, no. 12, pp. 1338-1344, 2008.

[40] C. Gonzales-Gonzales, M. Garcia-Hoyos, J. Trujillo-Tiebas, A. Bustamente Aragones, M. Rodriguez de Alba, D. Diego Alvares, J. Diaz-Recasens, C. Ayuso and C. Ramos, "Improvement in strategies for the non-invasive prenatal diagnosis of Huntington disease," *Journal of Assisted Reproduction and Genetics,* vol. 25, pp. 477-481, 2008.

[41] M. Gonzales-Gonzales, M. Garcia-Hoyos, M. Trujillo, M. Rodriguez de Alba, J. Lorda-Sanchez, J. Diaz-Recasens, C. Ayuso and C. Ramos, "Prenatal detection of a cystic fibrosis mutation in fetal DNA from maternal plasma," *Prenatal Diagnosis,* vol. 22, no. 10, pp. 946-948, 2002.

[42] R. McCormick, R. Nelson, G. Alonso-Amigo, D. Benvegnu and H. Hooper, "Microchannel electrophoretic separations of DNA in injection-molded plastic substrates," *Analytical Chemistry,* vol. 69, no. 14, pp. 2626-2630, 1997.

[43] D. Duffy, C. McDonald, O. Schueller and G. Whitesides, "Rapidprototyping of microfluidic systems in poly(dimethylsiloxane)," *Analytical Chemistry,* vol. 70, no. 23, pp. 4974-4984, 1998.

[44] V. Goral, Y.-C. Hsieh, O. Petzold, R. Faris and P. K. Yuen, "Hot embossing of plastic micro-fluidic devices using poly(dimethylsiloxane) molds," *Journal of Micromechanics and Microengineering,* vol. 21, pp. 1214-1216, 2011.

[45] H. Becker and U. Heim, "Hot embossing as a method for the fabrication of polymer high aspect ratio structures," *Sensors and Actuators,* vol. 83, pp. 130-135, 2000.

[46] L. Klintberg, M. Svedberg, F. Nikolajeff and G. Thornell, "Fabrication of a paraffin actuator using hot embossing of polycarbonate," *Sensors and Actuators A,* vol. 103, pp. 307-316, 2003.

[47] S. Lan, H.-J. Lee, E. Kim, J. Ni, S.-H. Lee, X. Lai, J.-H. Song, K.-L. Nak and M. Lee, "A parameter study on the micro hot-embossing process of glassy polymerfor pattern replication," *Microelectronic Engineering,* vol. 86, pp. 2369-2374, 2009.

[48] S.-J. John Lee and N. Sundararajan, Microfluidics Fabrication Handbook, Artech House, 2010.

[49] H. Klank, J. Kutter and O. Geschke, "CO2 laser micromachining and back end processing for rapid production of PMMA-based microfluidic systems," *Lab on a Chip,* vol. 2, pp. 242-246, 2002.

[50] M. Roberts , J. Rossier, P. Bercier and H. Girault, “UV laser machined polymer substrates for the development of microdiagnositc systems,” *Analytical Chemistry,* vol. 69, pp. 2035-2042, 1997.

[51] W. Pfleging, R. Kohler, P. Schierjott and W. Hofmann, “Laser patterning and packaging of CCD-CE-Chips made of PMMA,” *Sensors and Actuators B: Chemical,* vol. 138, pp. 336-343, 2009.

[52] J.-Y. Cheng, C.-W. Wei, K.-H. Hsu and T.-H. Young, “Direct-write laser micromachining and universal surface modification of PMMA for device development,” *Sensors and Actuators B,* vol. 99, pp. 186-196, 2004.

[53] F. Tagliaro, G. Manetto, F. Crivellente and F. Smith, “A brief introduction to capillary electrophoresis,” *Forensic Science International,* vol. 92, pp. 75-88, 1998.

[54] V. Dolnik, “DNA sequencing by capillary electrophoresis,” *Journal of Biochemical and Biophysical Methods,* vol. 41, pp. 103-119, 1999.

[55] H. Swerdlow, K. Dew-Jager, K. Brady, R. Grey, N. Dovichi and R. Gesteland, “Stability of capillary gels for automated sequencing of DNA,” *Electrophoresis,* vol. 13, pp. 475-483, 1992.

[56] M. Ruiz-Martinez, J. Berka, A. Belenkii, F. Foret, A. Miller and B. Karger, “DNA sequencing by capillary electrophoresis with replacable linear polyacrylamide and laser-induced flourescence detection,” *Analytical Chemistry,* vol. 65, pp. 2851-2858, 1993.

[57] P. Grossman , “Electrophoretic separation of DNA sequencing extension products using low-viscosity entangled polymer networks,” *Journal of Chromatography A,* vol. 663, pp. 219-227, 1994.

[58] S. Bagha, R. Chambers and J. Santiago, “Coupled Isotachophoretic Preconcentration and Electrophoretic separation using bidirectional isotachophoresis,” *Analytical Chemistry,* vol. 83, pp. 6154-6162, 2011.

[59] T. Herskovits, “Nonaqueous Solutions of DNA; Denaturation by Urea and Its Methyl Derivatives,” *Biochemistry,* vol. 2, pp. 335-340, 1963.

[60] M. Rocheleau, R. Grey, D. Chen, H. Harke and N. Dovichi, “Formamide modified polyacrylamide gels for DNA sequencing,” *Electrophoresis,* vol. 13, no. 8, pp. 484-486, 1992.

[61] P. Lindberg and J. Roeraade, “Non-aqueous gel matrices for separation of DNA fragments,” in *HPCE ‘96, 8th International Symposium on High Performance Capillary Electrophoresis*, 1996.

[62] B. Rosenblum, F. Oaks, S. Menchen and B. Johnson, “Improved single-stranded DNA sizing accuracy in capillary electrophoresis,” *Nucleic Acids Research,* vol. 25, pp. 3925-3929, 1997.

[63] T. Nock, J. Dove, B. McCord and D. Mao, “Temperature and pH studies of short tandem repeat systems using capillary electrophoresis at elevated pH,” *Electrophoresis,* vol. 22, pp. 755-762, 2001.

[64] Y. Kim and E. Yeung, “Separation of DNA sequencing fragments up to 1000 bases by using poly(ethyleneoxide)-filled capillary electrophoresis,” *Journal of Chromatography A,* vol. 781, pp. 315-325, 1997.

[65] O. Muller, M. Minarik and F. Foret, “Ultrafast DNA analysis by capillary electrophoresis/laser induced fluorescence detection,” *Electrophoresis,* vol. 19, pp. 1436-1444, 1998.

[66] F. Tagliaro, W. Smyth, S. Turrina, Z. Deyl and M. Marigo, “Capillary electrophoresis: a new tool in forensic toxicology. Applications and prospects in hair analysis for illicit drugs,” *Forensic Science Intemational,* vol. 70, pp. 93-104, 1995.

[67] E. Jellum, H. Dollekamp and C. Blessum, “Capillary electrophoresis for clinical problem solving: analysis of urinary diagnostic metabolites and serum proteins,” *Journal of Chromatography B,,* vol. 683, pp. 55-65, 1996.

[68] J. Petersen, A. Okorodudu, A. Mohammad and D. Payne, “Capillary electrophoresis and its application in the clinical laboratory,” *Clinica Chimica Acta,* vol. 330, pp. 1-30, 2003.

[69] A. Plecis, R. Schoch and P. Renaud, “Ionic transport phenomena in nanofluidic: experimental and theoretical study of the exclusion-enrichment effect on a chip,” *Nano Letters,* vol. 5, no. 6, pp. 1147-1155, 2005.

[70] R. Dhopeshwarkar, R. Crooks, D. Hlushkou and U. Tallarek, “Transient Effects on Microchannel Electrokinetic Filtering with an Ion-Permselective Membrane,” *Analytical Chemistry,* vol. 80, pp. 1039-1048, 2008.

[71] S. Pennathur and J. Santiago, “Electrophoresis in nanochannels,” in *ASME Joint U.S. - European Fluids Engineering Summer Meeting*, Miami, Florida, 2006.

[72] F. Baldessari and J. Santiago, “Electrophoresis in nanochannels: brief review and speculation,” *Journal of Nanobiotechnology,* vol. 4, no. 12, 2006.

[73] F. Leinweber and U. Tallarek, “Concentration polarization-based nonlinear electrokinetics in porous media: induced-charge electroosmosis,” *The Journal of Pysical Chemistry B,* vol. 109, no. 46, pp. 21481-21485, 2005.

[74] J. Benavente and R. de Lara, “Modification of a PET membrane/solution interface: effect of electrical parameters,” *Portugaliae Electrochimica Acta,* vol. 25, pp. 79-88, 2007.

[75] T. Hahn, C. O Sullivan and K. Drese, “Microsystem for field amplified electrokinetic trapping preconcentration of DNA at poly(ethylene teraphthalate) membranes,” *Analytical Chemistry,* vol. 81, no. 8, pp. 2904-2911, 2009.

[76] V. Kasicka, “Recent advances in CE and CEC of peptides (2007-2009),” *Electrophoresis,* vol. 31, no. 1, pp. 122-146, 2010.

[77] F. Foret, “Capillary electrophoresis of small ions using complex formation and indirect detection,” *Electrophoresis,* vol. 30, no. 1, pp. S34-S39, 2009.

[78] U. Pyell, “Characterization of nanoparticles by capillary electromigration separation techniques,” *Electrophoresis,* vol. 31, no. 5, pp. 814-831, 200.

[79] A. Jouyban and E. Kenndler, “Impurity analysis of pharmaceuticals using capillary electromigration methods,” *Electrophoresis,* vol. 29, no. 17, pp. 3531-3551, 2008.

[80] J. Prest, S. Baldock, P. Fielden, N. Goddard and B. Treves Brown, “A miniaturized isotachophoresis method for magnesium determination,” *Analytical and Bioanalytical Chemistry,* vol. 394, no. 5, pp. 1299-1305, 2009.

[81] M. Kurzawa, A. Jastrzebska and E. Szlyk, “Application of isotachophoretic and conductometric methods for neomycin trisulphate determination,” *Chemical papers,* vol. 63, no. 3, pp. 255-260, 2008.

[82] Z. Xu, T. Hirokawa, T. Nishine and A. Arai, “High-sensitivity capillary gel electrophoretic analysis of DNA fragments on an electrophoresis microchip using electrokinetic injection with transient isotachophoretic preconcentration.,” *Journal of Chromatography A,* vol. 990, no. 1-2, pp. 53-61, 2003.

[83] T. Hirokawa, Y. Takayama, A. Arai and Z. Xu, “Study of a novel sample injection method (floating electrokinetic supercharging) for high-performance microchip electrophoresis of DNA fragments,” *Electrophoresis,* vol. 29, pp. 1829-1835, 2008.

[84] D. Liu, B. Chen, L. Wang and X. Zhou, “On-chip coupling of free-solution transient ITP and CGE for highly efficient separation of dsDNA with variable sample loading amounts,” *Electrophoresis,* vol. 30, pp. 4300-4305, 2009.

[85] A. Rios, M. Zougagh and M. Avila, “Miniaturization through lab-on-a-chip: Utopia or reality for routine laboratories? A review,” *Analytica Chimica Acta,* vol. 740, pp. 1-11, 2012.

[86] A. Manz, N. Graber and H. Widmer, “Miniaturised total chemical analysis systems: a novel concept for chemical sensing,” *Sensors and actuators,* vol. B1, pp. 244-248, 1990.

[87] S. Julich, M. Riedel, M. Kielpinski, M. Urban, R. Kretschmer, S. Wagner, W. Fritzsche, T. Henkel, R. Moller and S. Werres, "Development of a lab-on-a-chip device for diagnosis of plant pathogens," *Biosensors and Bioelectronics,* vol. 26, pp. 4070-4075, 2011.

[88] R. Walczak, P. Sniadek, J. Dziuban, B. Kempisty, M. Jackowska, P. Antosik and J. Jaskowski, "Lab-on-a-chip spectrophotometric characterization of porcine oocytes," *Sensors and Actuators B: Chemical,* vol. 165, pp. 38-43, 2012.

[89] A. Testouri, L. Arriaga, C. Honorez, M. Ranft, J. Rodrigues, a. van der Net, A. Lecchi, A. Salonen, E. Rio, R. Guillermic, D. Langevin and W. Drenckhan, "Generation of porous solids with well-controlled morphologies by combining foaming and flow chemistry on a Lab-on-a-Chip," *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* vol. 413, pp. 17-24, 2012.

[90] S. Mouradian, "Lab-on-a-chip: applications in proteomics," *Current Opinion in Chemical Biology,* vol. 6, pp. 51-56, 2001.

[91] W. Kubicki, R. Walczak and J. Dziuban, "Miniature instrument for lab-on-a-chip capillary gel electrophoresis of DNA utilizing temperature control technique," *Procedia Engineering,* vol. 25, pp. 1237-1240, 2011.

[92] W. e. a. Kubicki, "Preliminary Studies on Cell-free Fetal DNA Separation and Extraction in Glass Lab-on-a-chip for Capillary Gel Electrophoresis," *Procedia Engineering,* vol. 47, pp. 1315-1318, 2012.

[93] M. Worgull, Hot Embossing: Theory and Technology, Burlington: Elsevier, 2009, pp. 70-71.

[94] C. Khan-Malek, "Laser processing for bio-microfluidics applications (part II)," *Analytical and Bioanalytical Chemistry,* vol. 385, pp. 1362-1369, 2006.

[95] S. Singh, "Lasers: Fundamentals, Types, and Operations," in *Nanomaterials: Processing and Characterization with Lasers*, Wiley-VCH Verlag GmbH & Co, 2012.

[96] W. Silfvast, Laser Fundamentals, Cambridge University Press, 2004, pp. 511-516.

[97] J. Giddings, Unified Separation Science, John Wiley and Sons, 1991.

[98] T. Jones, Electromechanics of Particles, Cambridge: Cambridge University Press, 1995.

[99] M. Hughes, "Strategies for dielectrophoretic separation in laboratory-on-a-chip systems," *Electrophoresis,* vol. 23, pp. 2569-2582, 2002.

[100] M. Kovarik and S. Jacobson, "Integrated Nanopore/Microchannel Devices for ac Electrokinetic Trapping of Particles," *Analytical Chemistry,* vol. 80, pp. 657-664, 2008.

[101] D. Hlushkou, R. Dhopeshwarkar, R. Crooks and U. Tallarek, "The influence of membrane ion-permselectivity on electrokinetic concentration enrichment in membrane-based preconcentration units†," *Lab on a Chip,* vol. 8, pp. 1153-1162, 2008.

[102] H.-C. Chang and G. Yossifon, "Understanding electrokinetics at the nanoscale: A perspective," *Biomicrofluidics,* vol. 3, pp. 012001-012001-012001-012014, 2009.

[103] R. Scott, "Isotachophoresis," [Online]. Available: http://physicalchemistryresources.com/ISOTACHOPHORESIS2.pdf. [Accessed November 2012].

[104] D. Liu, Z. Ou, M. Xu and L. Wang, "Simplified transient isotachophoresis/capillary gel electrophoresis method for highly sensitive analysis of polymerase chain reaction samples on a microchip with laser-induced fluorescence detection," *Journal of Chromatography A,* vol. 1214, pp. 165-170, 2008.

[105] J. Garcia de la Torre, M. Lopez Martinez and M. Tirado, "Dimension of short, rodlike macromolecules from translational and rotational diffusion coefficients. Study of the gramisidin dimer," *Biopolymers,* vol. 23, pp. 611-615, 1984.

[106] Y. Lu and N. Stellwagen, "Monovalent cation binding by curved DNA molecules containing variable numbers of a-tracts," *Biophysical Journal,* vol. 94, no. 5, pp. 1719-1725, 2008.

[107] S. Quake, "The Zimm model applied to extended single polymers," *Journal of Chemical Physics,* vol. 101, no. 5, pp. 4307-4311, 1994.

[108] S. Quake, H. Babcock and S. Chu, "The dynamics of partially extended singlemolecules of DNA," *Nature,* vol. 388, pp. 151-154, 1997.

[109] E. Stellwagen and N. Stellwagen, "Determining the electrophoretic mobility and translational diffusion coefficients of DNA molecules in free solution.," *Electrophoresis,* vol. 23, no. 16, pp. 2794-2803, 2002.

[110] D. Skoog, Principles of Instrumental Analysis, Belmont: Thomson, Brooks/Cole Publishing, 2007.

[111] C. Lu, D. Tso, T. Yang, Y. Jong and Y. Wei, "Detection of DNA mutations associated with mitochondiral diseases by Agilent 2100 bioanalyser," *Clinica Chimica Acta,* vol. 318, no. 1-2, pp. 97-105, 2002.

[112] "Agilent Technologies," Agilent Technologies, November 2012. [Online]. Available: http://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Product&SubPageType=ProductData&PageID=1636. [Accessed November 2012].

[113] D. Lambert and D. Draper, "Denaturation of RNA secondary and tertiary structure by urea: simple unfolded state models and free energy parameters account for measured m-values," *Biochemistry,* p. In Press, 2012.

[114] F. Ke, X. Mo, R. Yang, Y. Wang and D. Liang, "Polymer mixtures with enhanced compatibility and extremely low viscosity used as DNA separation media," *Electrophoresis,* vol. 31, pp. 520-527, 2010.

[115] H. Wei, C. Qi, X. Xu, Z. Zhang, Y. Zhou, Z. Cui, X.-E. Zhang and C. Zhang, "Glycerol-salt mediated stacking of nucleic acids in CZE," *Chromatographia,* vol. 67, pp. 491-494, 2008.

[116] E. Buel, M. LaFountaion and M. a. W. M. Schwartz, "Evaluation of Capillary Electrophoresis Performance Through Resolution Measurements," *Journal of Forensic Science,* vol. 46, no. 2, pp. 341-345, 2001.