Highly Scalable Combinatorial Mixing of Samples with Target-Specific Primers for Rapid Pathogen Detection on a Centrifugal Platform

Danielle Chung, B.Sc. (Hons)

School of Physical Sciences

Dublin City University

A thesis submitted to Dublin City University for the Degree of

Master of Science

Research Supervisor

Prof. Jens Ducrée

September 2016

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science, is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____

ID No.: 59518100

Date: _____

Acknowledgments

There are so many people I would like to thank throughout the duration of my time spent here. I'm sure I will have forgotten a few people and for that I hope you all know how much I appreciate your advice and friendship over the last few years.

First of all I would like to thank Prof. Jens Ducrée for giving me the opportunity to work within his very talented group and for the advice during my Master's degree. I would also like to acknowledge the support of the European Union (FP7-KBBE-2013-7-613908-Decathlon).

I would like to give the biggest thank you to all in the Microfluidics group, both past and present, which have supported me along the way to where I am now. I would like to thank Macdara who provided me with incredible advice and knowledge while I was here as an undergraduate student, Rohit and Damien who have always been more than happy to help with any question and to Lorcan who knows how to fix everything! You have all been a pleasure to work with and I thank you all so much. I would especially like to thank Tríona who was always around when I needed help, a rant, a cake recipe, a coffee break and chicken wings. I am forever grateful to this woman and wish her all the best in the future. I would like to give an extra special thanks to Dave Kinahan who has had to put up with me for the last few years. Dave has provided me with so much knowledge during my time here and I cannot thank him enough for that. My Masters has really been an enjoyable experience and Dave has definitely been a huge part of that.

I would like to thank all of the wonderful students who worked with me during their internships in the Microfluidics group. I am forever grateful for their dedication and hard work. I would like to give a special thanks to Artur and Simon who had the pleasure of building the "Monster Disc". These two students have a very bright future ahead of them and I would like to wish them all the best going forward.

A big thank you to everyone in the Biomedical Diagnostics Institute who I worked with during my time in DCU, especially those who had to endure listening to my frustrations over the last few years.

I would like to give my sincerest thanks to all the members of the DCU School of Physics who had a part in my time in DCU. I would especially like to thank Prof. Enda McGlynn and Prof. Colette McDonagh for always keeping me calm and for providing me with the best advice.

I would like to thank all of the postgrads who have kept me sane throughout my entire time in DCU. I have definitely made friends for life and I am so thankful to know all of you fantastic people. There are too many people to mention but I will mention Jen, Dan, May, Faith, Albina, Stephen and Paul. I would especially like to thank the genius that is Ivan for his incredible knowledge about absolutely everything! I have had some laughs with this man and I know that he is destined for only great things whether it is in the field of science or if someday I'll be singing to one of his songs on the radio. Most importantly I have to

thank my best hons Niamh and Éadaoin. These gals have been the reason why I loved coming into DCU every day. I knew I could always depend on these two for a happy cow, 'bucks and a great catch up. I have made friends for life in these two amazing people and I know that they will do incredibly in their future endeavours.

My best friends have been incredibly supportive over the past few years and I would like to sincerely thank them for that. I especially have to thank Joyce, Leanne, Becky and Ian who have managed to survive living with my breakdowns. I am surprised you are still my friends after it all!

To Ciarán, who has been the most supportive person along every step of the way. Thank you for being there through all the laughter and the tears, for picking me up when I've been down and for being the most kind and loving person. I could not have done this without you by my side.

And most of all I would like to thank my family. I would like to thank my sister Katie who has always been there for me and who has always supported me in everything I do. To my mam Anne who has believed in my abilities even when I had lost belief in myself. I could not have done this without their support and for that I am forever grateful. Thank you both so much.

A huge thank you to everyone x

Table of Contents

Chapter 3:

Chapter 4:

Glossary

BOTY	Botrytis Cineria
BSA	Bovine Serum Albumin
CAD	Computer-aided Design
cDNA	Complementary DNA
cSNP	Complementary Single-nucleotide Polymorphism
DF	Dissolvable Film
DI	Deionised
DNA	Deoxyribonucleic Acid
DXF	Drawing eXchange Format
EDTA	Ethylenediaminetetraacetic Acid
EHEC	Enterohaemorrhagic E.coli
GMO	Genetically Modified Organism
HC	Haemorrhagic Colitis
LAMP	Loop-mediated Isothermal Amplification
LoaD	Lab-on-a-Disc
LOC	Lab-on-a-Chip
LUO	Laboratory Unit Operations
miRNA	Micro RNA
mRNA	Messenger RNA
NA	Nucleic Acid
NTC	Non-template Control
PC	Poly(carbonate)
PCR	Polymerase Chain Reaction
PDMS	Poly(dimethlsiloxane)
PMMA	Poly(methlmethacrylate)
POC	Point-of-Care
PS	Poly(styrene)
PSA	Pressure Sensitive Adhesive

qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SNP	Single-nucleotide Polymorphism
TAE	Tris-acetate-EDTA
TYLCV	Tomato Yellow Leaf Curl Virus
UV	Ultra Violet

Nomenclature

Symbol	Description	Units
F	Force	N
F_C	Coriolis Force	N
F_E	Euler Force	N
F_ω	Centrifugal Force	N
P_{ABS}	Absolute Pressure	Pa
P_{ATM}	Atmospheric Pressure	Pa
P_{CRIT}	Critical Pressure	Pa
ΔP	Pressure Head	Pa
g	Acceleration due to gravity	m s^{-2}
h	height of fluid column	m
r	Radial distance from centre of rotation	m
r_0	Radial distance of the inner point of the fluid element from the centre of rotation	m
r_1	Radial distance of the outer point of the fluid element from the centre of rotation	m
Δr	Radial height of the fluid element (r_1-r_0)	m
$\bar{r}$	Mean radial distance of the fluid element from the centre of rotation	m
v	Fluid Velocity	m s^{-1}
ρ	Mass Density	Kg m^{-3}
ω	Angular Velocity of the disc	rad s^{-1}
ω_c	Frequency of rotation of the disc at which the valves are expected to open	Hz
$d\omega/dt$	Rotational Acceleration	rad s

List of Figures

List of Tables

Abstract

Highly Scalable Combinatorial Mixing of Samples with Target-Specific Primers for Rapid Pathogen Detection on a Centrifugal Platform

Danielle Chung

The capability to screen a large number of samples (M) for specific responses to a library of active agents (N) in a manner which is time- and cost- efficient is of critical importance in application areas such as plant diagnostics, crop genotyping and drug discovery. There is great interest in these areas for the identification of specific genes or plant pathogens in crops using DNA markers, DNA traceability for food safety and identification of a specific response of cells to a specific drug. DNA-based methods in the field of point-of-use devices are critical for on-site testing of samples without expensive instrumentation. However, the high cost of reagents and liquid handling robots required to perform vast numbers of pipetting steps significantly hampers the proliferation of key enabling technologies into smaller laboratories. Centrifugal microfluidic devices have emerged as increasingly useful tools for biomedical applications and diagnostics and can be manufactured using inexpensive materials and low cost instrumentation. The integration of DNA amplification methods are used to rapidly generate large amounts of DNA to reliably detect and identify diseases present in plant material which cause economic losses to agribusiness each year. This work demonstrates three microfluidic centrifugal platforms for automating the combinatorial mixing challenge in a simple instrument and demonstrates a reduction of pipetting steps towards large numbers of samples and reagents. These platforms permit M×N combinatorial mixing to generate unique sample/reagent outputs in an autonomous manner. These platforms demonstrate highly scalable automation of the liquid handling protocols required for combinatorial screening methods on a simple, spindle-motor based instrument. Furthermore, by significantly reducing the number of pipetting steps, by lowering reagent costs through miniaturisation as well as by accurate metering and widely eliminating human error in liquid handling, our technology meets the requirements of deployment in decentralized settings.

Chapter 1

Introduction

1.1 Introduction

Detection and identification of foodborne pathogens has been of great interest for the food industry and regulatory community, particularly for ensuring the integrity of the food supply chain. Food diagnostic testing is an important tool for early detection and identification of potential pathogen outbreaks; thus ensuring safe food production and minimal impact on public health and wellbeing. Fast identification and quick preventative action can significantly reduce the adverse health effects and economic cost associated with pathogen contaminated food.

Foodborne pathogens, such as *Escherichia coli* O157:H7 and *Salmonella sp,* can colonise the intestinal tracts of healthy animals in the food chain and can live on healthy plants, have the ability to spread to a variety of foods which are used for human consumption. Pathogenic infections which go undiagnosed and unreported result in millions of illnesses and thousands of deaths worldwide each year [1]. Due to modern practises, such as long supply chains for industrial production, and due to increased travel and trade, there is an ever increasing risk of food contamination [2]. Although measures such as removing contaminated products from a shelf can limit an immediate outbreak, it is critical that the source of contamination be determined to ensure that similar outbreaks do not occur in other, often geographically distant, locations which share the same food supply [3].

In addition to the potential direct impact on human health, foodborne pathogens also have devastating effects on agricultural and food supplies. Vegetable crops are often affected by microorganisms including fungal and bacterial species and these incur huge economic losses for farmers and agribusiness. With the help of other organisms, these fungal and bacterial agents have the ability to enter the plant tissue through wounds caused by chilling or mechanical injuries. As well as directly damaging the crop resulting in decreased crop development and crop losses, some fungal species can produce toxic metabolites which in turn cause potential health risks to humans, for example the hepatoxic aflatoxin produced by *Aspergillus flavus*. Plants physiological properties such as high water availability and nearly neutral pH make them a very attractive platform for microbial growth and infection. Some microorganisms including bacterial agents (*Erwinia carotovora*) and fungal agents (*Botrytis cineria*), are weak plant-pathogens and start the decomposition process before harvest. In turn, vegetables are particularly vulnerable after harvest as they may be expected to contain high numbers of microorganisms due to contact with soil during growth. Spoilage to vegetables, caused by bacteria and fungi, is due to optimal conditions for this microbial growth. Other contamination occurs after harvesting during transportation, processing and/or storage [4]. One potential contaminant, Whiteflies, which are insects with the ability to transmit pathogenic diseases, is addressed during this project and has caused major problems to vegetable crops resulting in significant economic losses. Economic losses are incurred due to the plants physiological processes being affected due to high feeding populations. This causes leaf shredding and crops have showed a reduction in growth rate which is detrimental to the livelihood of farmers [5].

As previously mentioned to minimise risk of infection for the consumer, microbial quality control programs are increasingly put in place during food chain production. The use of detection systems to determine the presence or absence of pathogenic contaminants has

therefore become increasingly important throughout the agricultural and food industry. The current gold standard detection method involves the enrichment and isolation of colonies of fungi or bacteria on a solid media. After culturing to enrich the samples, biochemical and/or serological identification are carried out. Although these traditional methods are reliable and well characterised, they may require several days or weeks of analysis to obtain results. Similarly, they require a significant support infrastructure including laboratories and skilled technicians. Often, identification can resolve to a particular bacteria species but not its serotype. Thus, they are not very compatible with point-of-use testing in a distributed manufacturing industry. The slow time-to-answer means these methods are not very practical for providing food safety authorities, in the event of a pathogen outbreak, with data to make timely interventions.

One alternative method for the rapid detection and identification of food pathogens involves testing samples for the deoxyribonucleic acid (DNA) of a potential contaminating microorganism. As the amount of pathogen DNA is usually below measurable levels by non-PCR methods, a method of DNA amplification, such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) for DNA amplification [6][7][8][9][10][11][12], are often used. PCR is an extremely powerful diagnostic tool for the analysis of microbial infections and microorganisms in food samples and has been established as an alternative detection method in microbial diagnostics. Advantages of using this method include good detection limit, specificity, sensitivity, selectivity and the production of results in a short period of time. Another major advantage is the potential for integration into portable and highly automated systems [13]. However, there are also limitations regarding this method of DNA amplification. Although PCR is highly sensitive, trace amounts of DNA can cause sample contamination and produce misleading results.

Prior sequence data is also required to design primers for PCR; thus PCR can only be used to identify the absence or presence of a known gene or pathogen [14].

Loop-mediated isothermal amplification (LAMP) is an emerging method of DNA amplification. Recently, LAMP has been used as an alternative to PCR-based methods in food testing and in a wide range of other applications. A number of advantages LAMP presents over PCR-based methods include: shorter reaction time; higher sensitivity and specificity; and no requirement for specialised equipment. In addition there is low susceptibility to inhibitors present in sample materials compared to PCR-based methods. As this method is isothermal, and therefore does not require temperature cycling, it is particularly suitable for 'point-of-use' detection devices. This is particularly the case as the heating and cooling required for PCR can make portable devices inefficient and power hungry. LAMP has been used to detect many important foodborne pathogens including bacteria and viruses including *E.Coli and Salmonella* [15]. Industrial laboratories and public health sectors are beginning to implement this DNA amplification method and to use it in parallel with official protocols [16]. DNA amplification methods are further described later in this Section 1.4.

In recent years microfluidic platforms have been used for the development of diagnostic tools. Microfluidics is commonly defined as the science and technology that processes minute volumes of fluids using channels with dimensions in the tens to hundreds of micrometres [17]. They provide small, cheap and integrated technologies, whose fluid handling capabilities makes them an ideal option to carry out a large range of diagnostic tests. Thus tests which are commonly carried out on laboratory equipment which are often bulky, expensive, and require skilled operators for analysis, can be performed by a low-skilled user in the field / at point-of-sampling.

Applications of microfluidic devices include the detection of pathogens and toxins in food, water supply monitoring for the presence of residues and trace chemicals, monitoring nutrients and water supplies on farms to improve efficiency in fertilisation, and for plant cell sorting to improve crop production [18]. The integration of microfluidic devices in all parts of agri-business, and particularly in food diagnostics, can have a major impact on world food security. In particular, providing pathogen detection at the source of contamination can greatly reduce adverse health effects in the general public.

A common application of genetic testing is screening. Here, a sample is tested against a number of active agents for a response. In the case of point-of-use food testing, a single sample might be genetically screened against a series of markers to indicate if any of these pathogens are present. On the larger scale, screening of multiple samples against multiple active agents can be used in a laboratory environment for application such as food traceability, pathogen detection, Genetically Modified Organism (GMO) detection and, in the non-food domain, for forensic testing and human-disease diagnostics.

1.2 Genetic Screening

In the medical context, screening can be defined as the process of systemic testing of asymptomatic individuals for pre-clinical disease. For example, to prevent or delay the development of disease through early detection and effective treatment [19]. However, screening is not only limited to individuals for the detection of genetic diseases. More generally, screening can be defined as detecting the response of a sample to a particular active agent. Screening is widely used in the agricultural and food industry to detect the food pathogens that can affect food supply chains. Such screening applications permit the detection of the source of a pathogenic contaminant which would have devastating effects

on agricultural industry and for public health. These screening applications could also be used to detect GMOs which might be unregulated and illegal in some legislatures.

An important method of genetic screening, used widely for applications such as disease research, is genotyping. Genotyping is a screening process whereby a DNA sequence is compared to a reference sequence. DNA is a biological molecule essential for all forms of life. Valuable information is stored in DNA which is one of the key elements to determine genetic disorders or pathogenic diseases in the field of human or food diagnostics. By comparing an individual sequence to a reference library, variations of this sequence from the norm, called single-nucleotide polymorphisms (SNPs), can be identified. Many of these SNPs can be associated with particular traits, ranging in humans from eye colour to pre-disposition to cancers. Similarly, in plants SNPs can determine if a plant will grow in an arid environment or have immunity to common pests. In the case of food bacterial contaminants, SNPs can be used to identify a particular sub-type or variant (serotype) of bacteria (for example Shiga toxin 1 *E. coli* vs Shiga toxin 2 *E. coli*). This can be used to prevent contaminants entering the food supply and, in the event of an outbreak, support epidemiologists trace the source of contamination and support clinicians in treating patients who have been affected by the pathogen.

SNPs are the most frequent type of variation in the human genome. SNPs are a variation in a single nucleotide in a DNA sequence and can develop in a number of different ways [20]. Complementary DNA (cDNAs) (cSNPs) are commonly classed as SNPs and reflect underlying genomic variants [20]. Inefficient replication of DNA during cell division is one cause of SNPs. Exposure to external factors, including radiation, which causes a breakdown of DNA can also cause SNPs to occur [21]. Where these variants are found in DNA sequences between the genes (so called junk DNA) they have minimal effects but can act as biological

markers for applications such as forensic testing or, as previously described, food traceability and food pathogen identification.

Where a SNP occurs within an active gene, it can in some cases vary the behaviour of this gene which, alone or in combination with other SNPs, can trigger diseases including some cancers. A person born with inherited SNPs might therefore be statistically more likely to develop diseases than someone who is not born with these SNPs present. Thus the detection of SNPs is particularly useful for genetic screening of the human genome. It is only recently been used the detection of foodborne pathogens or for the detection of GMOs. Most genotyping, including the detection of pathogenic diseases, is based on the amplification of particular regions of the DNA using techniques which will be discussed in detail in the Section 1.4 [22].

One of the major requirements of genetic screening is the capability to screen a large number of samples for specific responses to a library of active agents. One of the most important aspects to consider is the time and cost efficiency of these systems to carry out such screening methods. In application areas such as plant diagnostics, pharmaceuticals, forensics and genotyping, time and cost efficient screening methods are of growing interest and are developing constantly to provide high standard DNA detection methods for analysis. It is for this reason that we have identified a microfluidics-based approach as a potential platform for enabling genotyping to be performed in a more cost-efficient and practical manner.

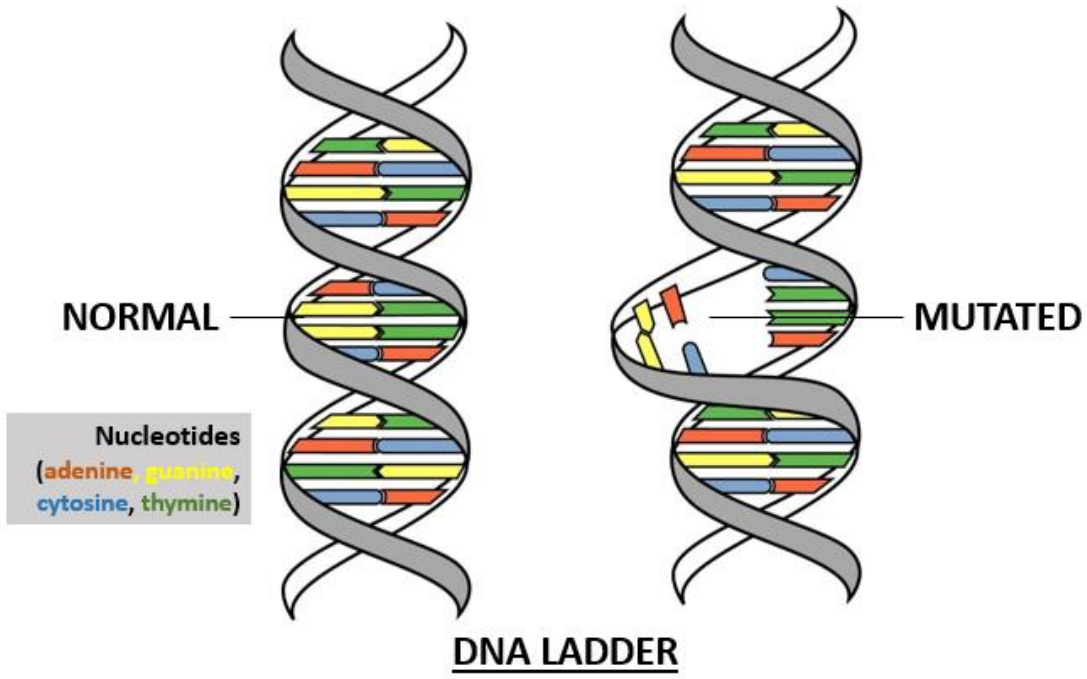

Figure 1: DNA structure. Comparison of a normal DNA structure with a mutated DNA structure which shows a variation in nucleotides [23].

There have been increased requirements for rapid detection of foodborne pathogens in the agricultural and food industry. Avoiding economic losses due to crop devastation and ensuring a low level of pathogenic contaminants present in the food supply chain are of critical importance [24]. There have been many major developments in methods for rapid detection of foodborne pathogens to ensure reliable safe food supplies are available worldwide. Improvements in automation, molecular biology and immunology have positively improved sensitive and convenience of methods applied in food microbiology. Traditional culture-based methods are commonly known to be time-consuming and laborious which presents a requirement for rapid identification of foodborne pathogens. Recently there have been advances in molecular cloning and recombinant DNA techniques which have made a huge impact on the detection of pathogens in food. Such advances

include PCR-based techniques [25] and new bio-molecular techniques to improve biosensor technologies for rapid pathogen detection [26].

Despite these advances, agricultural and food industries remain extremely susceptible to pathogenic contamination and disease outbreaks as pathogens are present in foods, soil and water. This has resulted in increased numbers of reported deaths and illnesses [27] associated with pathogenic food outbreaks. One of the reasons more and more outbreaks of foodborne illnesses are recorded every year is the difficulty in detecting these outbreaks quickly and at the point-of-sampling. Better diagnostics for the detection of these outbreaks also contribute to the high levels of cases recorded each year. On-going changes in the food supply and the identification of new foodborne illnesses require more reliable and accurate guides to assess the effectiveness of food safety regulations [28]. The availability of 'big data' , the determination of the overall number of episodes of foodborne illnesses and their source, can be an incredibly useful tool for the allocation of resources to limit current outbreaks and prevent future outbreaks. This can allow prioritisation of resources to areas which require the implementation of a more rigorous food safety procedure for the prevention of foodborne pathogens. This will ensure contaminated food products are identified at early stages and not distributed to the public causing the spread of pathogenic diseases.

There are many ways in which food can become contaminated such as viruses, bacteria, chemicals and parasites. Ingestion of contaminated food products is the main transmission of foodborne pathogenic diseases. Transmission can also occur through non-food mechanisms such as coming into contact with animals and contaminated water. Not all individuals react in the same manner to the transmission of a foodborne pathogenic

disease. The age and immunity of an individual play a huge role in the proportion of the disease transmitted by food [1].

One foodborne pathogen of major interest is Escherichia coli O157:H7 which is an enterohaemorrhagic serotype of the bacteria *E. coli*. This strain of *E. coli* poses a worldwide threat to public health as it has been implicated in outbreaks of haemorrhagic colitis (HC). These outbreaks have caused fatalities by haemolytic uraemic syndrome [29]. There are approximately seventy-five thousand reported cases of E.coli O157:H7 infections across the United States recorded on an annual basis [28]. Common foods implicated in pathogenic foodborne outbreaks included undercooked poultry, seafood, meat, unpasteurised milk and organic salad vegetables [3]. As beef and unpasteurised dairy products are most commonly associated with enterohaemorrhagic e.coli (EHEC) infection it is suggested that cattle are natural reservoirs of such pathogenic diseases. Studies have revealed them as natural hosts of the bacterium which is then integrated into the food supply chain causing an outbreak of pathogenic illnesses [30]. As previously mentioned EHEC produce a number of symptoms which includes HC i.e. bloody diarrhoea [31]. When an individual is affected by contaminated food initial symptoms are often presented in the form of non-bloody diarrhoea. Over a few days this progresses into bloody diarrhoea accompanied by severe abdominal pain and mild dehydration. Diagnosis of HC is isolation detection and identification of EHEC in patient stool samples. The main treatment available to individuals affected by HC focuses on rehydration and supportive therapy [30]. The screening of food products intended for the food supply chain could detect and identify diseases such as EHEC at early stages to isolate contaminated food products ensuring they are not available for distribution.

Other foodborne pathogens of great interest are the pathogenic diseases of vegetable crops such as tomatoes. Crops can become infected by pathogenic diseases in a number of different ways. These include trade of plant materials which may introduce new viruses into production systems, increased international travel and changing climate conditions which can increase the spread of newly introduced viruses. Antiviral products are generally unavailable to control the spread of new viruses requiring farmers to rely on genetic resistance of crops or using hygienic measures to prevent viral diseases. Another prevention method is the eradication of diseased crops to ensure all traces of the crops are destroyed, preventing further distribution of the pathogen [2].

Two related pathogenic viral species, *Torradovirus genus,* have emerged in Spain and Mexico posing a threat to crops in the last decade. These identified viral species cause devastation to crops such as severe necrotic leaf symptoms observed in tomato plants. This was observed as a burn-like appearance on the affected leaves. The disease consisted of necrotic spots surrounded by a light green or yellow area at the base of the leaflets. As the disease progresses, later stages display severe necrosis in leaves and fruit resulting in overall growth reduction. This overall reduction in crop growth has major implications causing serious economic damage [32].

Another detrimental viral disease is the tomato yellow leaf curl (TYLCV) disease which is most commonly transmitted by an infestation of whitefly. In many regions the spread of TYLCV is the main limiting factor in tomato production. This has a major economic significance as entire crops are lost due to this infestation every year. This is a complex virus species causing up to 100 % economic losses to tomato crops in many tropical and subtropical regions worldwide [33]. Symptoms include the upward curling of leaflet margins, the reduction of leaflet area and young leaves displaying a yellow colour resulting

in stunted growth and flower abortion. This disease results in reduced yields and plant production is almost entirely lost if infection occurs at the early stages of plant growth [34].

The *Botrytis cinerea* (BOTY) pathogen is a necrotrophic fungus which causes damage to vegetables, fruits and cut flowers and causes a grey mould to form in a wide range of plant species. Particularly BOTY is a severe threat to field and greenhouse-grown tomatoes in many countries worldwide as countries with high humidity often stimulate the spread of the disease. This is particularly prone to infestation if the free moisture is present on the plant surface. The development of synthetic fungicides, one option is anilinopyrimidines, have controlled the disease over many years and have proven to be very effective [35]. However, the continued application of synthetic fungicides has disrupted biological control by natural predators and has led to outbreaks in disease and widespread resistance to various types of fungicides [36].

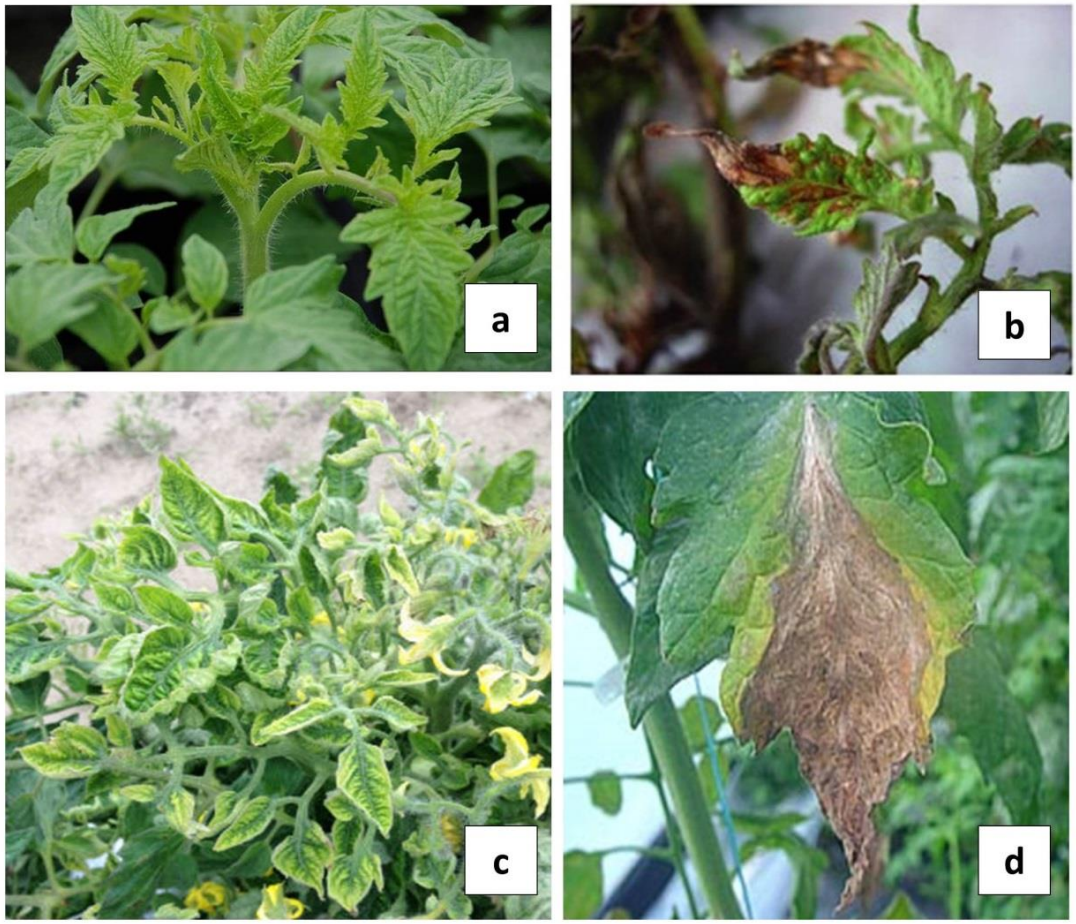

Figure 2: Affected tomato leaves. a) A healthy tomato leaf plant showing no sign of infection [37]*. b) Tomato leaf infected with Torrado Virus displaying damaged leaf tips* [32]*. c) Tomato leaf infected with TYLCV showing yellowing of the entire leaf structure* [38] *and d) Tomato leaf infected with BOTY displaying big areas of leaf decay* [39]*.*

Screening for the aforementioned foodborne pathogens has been increasingly developed over the last few years with advancements in Nucleic Acid (NA) based detection methods. Some of the most common NA detection methods are discussed in more detail in the Section 1.4.

1.3 Other Applications of Screening

Screening can be used in other areas including forensic; wildlife and agriculture; and medical applications.

The application of screening in the area of forensics is widely used based on DNA and RNA analysis. Ribonucleic acid (RNA) is a polymeric molecule implicated in gene expression and has gained increasing interest in forensic applications. Similar to DNA, the structure of RNA is observed as a chain of nucleotides. It differs to DNA as it is a single-strand folded onto itself. Messenger RNA (mRNA) conveys genetic information which synthesises specific proteins. Viruses commonly encode their genetic information using the RNA genome. Recently mRNAs have been used for tissue-specific expression in body fluid identification. mRNA profiling was proposed for forensic screening as it is a highly sensitive and specific method for detecting genetic variances [40]. mRNAs are particularly useful due to their durability to withstand long periods of storage in sun-protected, dry, and ambient-temperature conditions. mRNA markers with tissue-specific expression pattern are still detectable in biological stains under these conditions making them highly suitable for body fluid identification in forensic screening [41]. Although mRNAs are able to withstand the aforementioned conditions, degradation is expected when exposed to more aggressive environmental factors such as UV light, moisture and heat which are all relevant for forensic samples. Degradation of mRNA molecules causes physical fragmentation to occur and this dictates the use of short amplicons for successful PCR detection. However, micro RNAs (miRNAs), which are fundamentally short, have great potential for being ideal markers in forensic applications especially for forensic body fluid identification [42] miRNAs are non-protein coding RNA molecules consisting of eighteen to twenty-two nucleotides in length and negatively regulate gene expression. Zubakov *et al.* have demonstrated that

miRNAs have become very useful in identifying body fluids in forensic practice for crime scene reconstruction [42].

Another application of screening is in the area of wildlife and agriculture. Wildlife forensics is the application of genetic identification techniques providing evidence in wildlife crime investigations. The conservation of flora and fauna is of high concern and regulation is required for the conservation of species. This enables sustainable exploitation of natural resources. The Convention on the International Trade in Endangered Species (CITES) implements regulations aimed to achieve this. However, the implementation of these regulations is often faced with difficulties relating to identification of animals and plants or their products and product derivatives [43]. To regulate such aspects forensic genetic approaches are implemented to support trade monitoring and enforcement worldwide [44]. Analytical tests are highly dependent on the availability of molecular genetic markers. In model species research and their applications in molecular ecological studies SNPs have increased the potential for using SNP markers in wildlife forensics [45]. Developments in microsatellite-based methods for investigating individual identity, parentage and geographic origin in companion animals, livestock and wildlife forensics are continuing to progress. DNA sequence variation has also been recognised as a potential method for identifying species and in wildlife investigation [43]. Initially, considerable cost and effort was required for approaches regarding SNPs in wildlife species. This was carried out to generate low numbers of polymorphic SNPs. The discovery and development of SNPs has resulted in increased availability and reduced costs of high throughput sequencing. This provides great potential to increase the number of SNP markers made available to population genetic applications. Reference genomes have been well established in model species such as pigs, sheep, cattle and humans. Large libraries containing data of SNP

markers are now available which have led to developments in high-density genotyping and SNP chips [43].

Recently the area of antibody and protein arrays has had a high level of interest in medical applications. This is due to advances in areas such as genome sequencing, robotics, proteomics, bioinformatics and microelectronics. To understand disease processes there is an increasing interest in automating approaches to analyse proteins. The search for disease-specific proteins has significant value for genotyping applications. These proteins may possess protein markers in areas of interest such as medical, diagnostic and commercial or may serve as drug targets and their corresponding gene sequences. Interference with disease-causing genes or proteins can relieve symptoms experienced by an individual. Due to this the ability to identify such disease-causing genes or proteins is of great interest. Observation and monitoring anti-bodies contained in serum is one such example of protein arrays showing great potential in diagnostic applications. However, although it is a major advantage to analyse biological processes on the protein level challenges still arise. One such challenge is the ability to automate the analysis of thousands of proteins paired with no simple method of protein amplification such as PCR [46].

Another application of medical screening is the area of pharmacogenomics. Pharmacogenomics is based on an individual's specific response by the administration of a certain drug. The ultimate aim of the area of pharmacogenomics seeks to gain information from different molecular substrates such as DNA, RNA and proteins between patients. The use of pharmacogenomics aims to improve guide therapy by prescribing the "right" drug for the "right" patient by tailoring a drug regimen based on to each individual patient requirement. One of the main influences in drug sensitivity is variations in DNA.

Polymorphisms in DNA can influence drug sensitivity by the alteration of mechanisms through which a drug works. Another factor affecting drug sensitivity is the differences in gene expression among individuals within individual cancers [47]. A genotyping test is required to identify most or all mutations causing a significant impact on the expression of function of drug-metabolising enzymes, drug receptors and transporter proteins. One method is restriction fragment length polymorphism (RFLP) analysis which is used as a hallmark for analysis of known mutations. As a result of development and major advancements in the area of pharmacogenomics a high level of automation will be required due to an anticipated high volume of pharmacogenomics testing. This will result in a high screening load due to the testing of patient samples against a vast library of drugs [48].

1.4 Methods of Genetic Screening

Over the last few decades there have been major breakthroughs in the area of DNA detection and the development of genetic screening methods. As a result, gene analysis procedures have become a key factor in molecular diagnostics. DNA amplification methods are extensively used for widespread applications which include pharmaceuticals, forensics, plant diagnostics, biotechnology and crop genotyping [49]. PCR technology, along with other DNA signal and target amplification techniques, has been established as a reliable genetic screening method. Molecular diagnostic methods are important and crucial tools in molecular diagnostics where nucleic acid detection and amplification is of high importance [50]. In this section PCR and qPCR are described as the most common nucleic acid amplification techniques. LAMP and NASBA are the most commonly adopted for microfluidic purposes.

1.4.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is the most common nucleic acid amplification technique due to its simplicity and specificity. This method can amplify a single copy or a few copies of a DNA fragment by several orders of magnitude. Due to the long waiting times associated with traditional microbial culture methods, other forms of NA amplification techniques have emerged. These methods provide more efficient sample throughput and generation of sample data. The development of PCR techniques provides greater specificity as it relies on sequence-based hybridisation chemistry, resulting in the direct detection of target microbial DNA. This direct detection reduces the reliance on cultivation and large reagent usage which also significantly reduces the time in which samples can be analysed and to obtain results [51].

PCR is carried out using a varying range of temperatures in a single cycle. DNA denaturation occurs at a temperature of 94 ˚C which is applied for one minute. The primers are annealed as the temperature is briefly dropped to between 40 ˚C and 60 ˚C. This is then followed by increasing the temperature to 72 ˚C for sufficient time to permit primer extension to take place. The denaturation and annealing temperatures are the most critical. Failure to adequately reach the denaturation temperatures results in the failure of DNA melting and thus no DNA amplification occurs. Application of low temperatures can cause mispriming and amplification of non-target sequences whereas higher temperatures lead to a lack of annealing and an absence of amplification [49].

PCR amplifies short regions of DNA *in vitro* by a process which is primarily enzyme-driven. This involves the denaturation of DNA, primer annealing and extension/elongation by DNA polymerase. The amplification is carried out by DNA polymerase in the presence of two

oligonucleotide primers that allow these processes to take place. These primers bind to the opposite ends of the target DNA sequence and cause the target DNA sequence to duplicate as the polymerase proceeds across the region between the two primers and thus the duplicated DNA of the region of interest increases exponentially. By repeating this cycle, amplification as much as a million fold can be achieved [52].

The PCR amplification process is described in Figure 3.

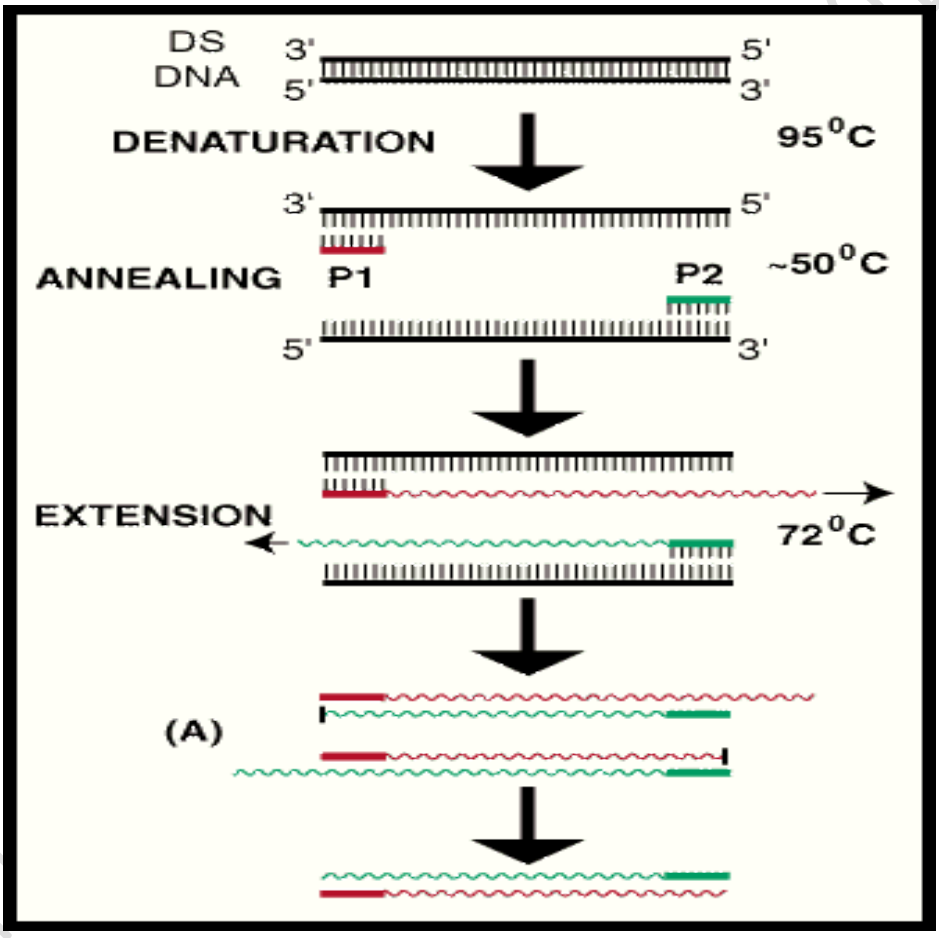

Figure 3: PCR amplification process [53]. *The denaturation step allows melting of the DNA template at 95 ˚C. The primers anneal to the single-stranded DNA template at ~ 50 ˚C during the annealing step. A new strand of DNA is synthesized which is complementary to the DNA template by the DNA polymerase at 72 ˚C during the extension step.*

Carrying out PCR has proven to be quite labour-intensive creating heavy workloads with multiple pipetting steps and further analysis of the amplified products. PCR involves careful

sample preparation procedures and bulky machines which is a limiting factor in deploying such a technique in the field for on-site testing. Such machines are often power hungry and require reliable power sources which are not always available for on-site testing or in poor-resource settings. Contamination of samples is a very common occurrence which also adds to the lengthy sample preparation as extreme caution must be used when preparation is taking place. Recently, the development of miniaturised PCR reactors have been integrated into microfluidic devices which dramatically decreases sample and reagent making them increasingly more accessible for use in resource poor settings [54]. As previously mentioned PCR requires three components for successful DNA amplification. In a screening process this would require three pipetting steps in each sample well for analysis. If numerous samples were being analysed this would increase the workload as the number of pipetting steps are increased. Therefore testing a number of samples (M) for a number of active agents (N) would significantly reduce the workload towards a more automated system. The ability to develop a combinatorial mixing system (MXN) would result in significantly less pipetting steps and would create various sample/reagents combinations for a more conclusive sample analysis.

1.4.2 Quantitative PCR (qPCR)

Quantitative PCR (qPCR), also known as real-time PCR, has the ability to continuously monitor PCR product formation throughout the process of the entire reaction. This method can offer simultaneous, rapid amplification and sequence-specific-based detection. One main advantage of using qPCR for nucleic acid amplification is no post-amplification analysis is required, such as gel electrophoresis, which significantly reduces the analysis time period [55]. qPCR permits detection of the PCR product from sample in real time, in addition to providing quantitative measurements and/or data.

The DNA product can be detected and quantified using two different methods; sequence-specific DNA probes which consist of fluorescently labelled reporter sequences; and fluorescent dyes that non-specifically intercalate with double-stranded DNA. Detection can only be carried out using these two methods after hybridisation of the probe with its complementary DNA target. Reverse transcription which permits mRNA to convert to cDNA can also be combined with qPCR. This allows quantification of the cDNA using qPCR and thereby indicating the amount of original mRNA in the sample [56].

DNA sequences can be analysed for mutations by differential hybridisation with sequence-specific oligonucleotide probes. The process of oligonucleotide analysis is based on specific hybridisation of a single-stranded nucleic acid oligonucleotide "probe" sequence with a sample "target" sequence to be detected [57]. Prior to hybridisation, PCR facilitates the use of probes to increase the number of copies of target DNA present in a sample. "Molecular beacons" are commonly used to carry out the detection of specific nucleic acids in living cells and homogenous assays. They are so called as they only emit a fluorescent signal when they are hybridised to target sequences. Probes are usually a single-stranded nucleic acid molecule which possesses a stem-loop structure. A probe sequence which is complementary to a predetermined sequence in a target nucleic acid is located on the loop portion of the molecule. The annealing of two complementary arm sequences located on either side of the probe sequence produces the stem. The arm sequences and target sequence are unrelated. One end of the arm contains the fluorescent element while the other end of the arm contains a non-fluorescent quenching element. These two elements are kept in close proximity by the stem which causes fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer. This fluorophore-quenching pair results in energy received by the fluorophore and being transferred to the quencher and dissipated as heat instead of emitting light. This results in the fluorophore being unable to

fluoresce. When a target sequence and a probe meet a hybrid is formed which is more stable and longer than one formed by the arm sequences. This causes the probe to undergo a conformational change, forcing the arm sequences to apart and resulting in the fluorophore and quencher to move apart from one another. The fluorophore fluoresces when exposed to UV light as it is no longer in close proximity to the quencher [58].

The detection of DNA is also carried out with fluorescent-based indicators such as fluorescent dyes that non-specifically intercalate with double-stranded DNA. The use of these dyes, such as SYBR green, has grown in popularity and are important for a number of analytic and diagnostic applications; detection of nucleic acids in gels; and in fluorescence imaging in qPCR [59]. Fluorescently-labelled DNA molecules emitting signals are detected using fluorescence detection systems including confocal microscopes. Usually the nucleic acid amplification method is employed before the detection assay is applied. Detection is carried out during the assay also, as a measurement is taken at the end of each cycle to produce a curve. Amplification techniques are commonly used to incorporate the fluorescent label. Fluorescently-labelled amplified target sequences are produced by the addition of fluorescent primers or nucleotides [57]. Reduced specificity of probes can also be dealt with by employed refined PCR techniques such as nested PCR. This refined PCR technique reduces non-specific binding in products caused by amplification of unexpected binding sites [60].

1.4.3 Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is one of the most common isothermal amplification methods which amplify DNA with high specificity and efficiency in a short period of time. As the name suggests, LAMP is performed using a single temperature for DNA amplification compared to PCR-based methods which requires temperature cycling. This makes LAMP an attractive method of DNA amplification for point-of-use testing as there is no requirement for expensive machines to create a multi-temperature environment. LAMP can be carried out using simple heating devices such as thermal heating blocks or air-heating guns to mimic the required isothermal conditions. LAMP is widely used for molecular diagnostics and food safety due to its cost-efficiency, rapid and specific diagnosis of various infectious diseases , including pathogens such as bacteria and viruses [16]. LAMP is also an ideal amplification method as it is less sensitive to substances that can inhibit standard PCR reactions.

There are three main steps in the LAMP process: initiation; cycling amplification step; and elongation. The reaction requires four-six specially designed primers, in addition to a DNA polymerase with strand displacement activity. The structure of the primers causes the replicated DNA sequences to produce a loop-like structure. These primers include two inner primers (FIP, BIP) and two outer primers (F3, B3). These primers recognise the six-eight primer regions within the target DNA. Due to amplification only occurring when all of the six regions within the target DNA are recognised by their corresponding primers this makes LAMP a highly specific method. Loop primers (LF, LB) which are designed to anneal at the loop-like structure may also be added to increase the sensitivity of the LAMP reaction.

There are a number of different ways in which LAMP amplification can be observed which include turbidity measurements, fluorescence measurements, and gel electrophoresis of

the LAMP reactions. These can be observed visually and quantitatively for a positive LAMP reaction making this a simplistic endpoint detection method [61].

Amplification of the target nucleic acid molecule using LAMP occurs at a temperature ranging between 60 ˚C and 65 ˚C within 60minutes [62]. At these temperatures the target can amplify up to one billion fold per hour.

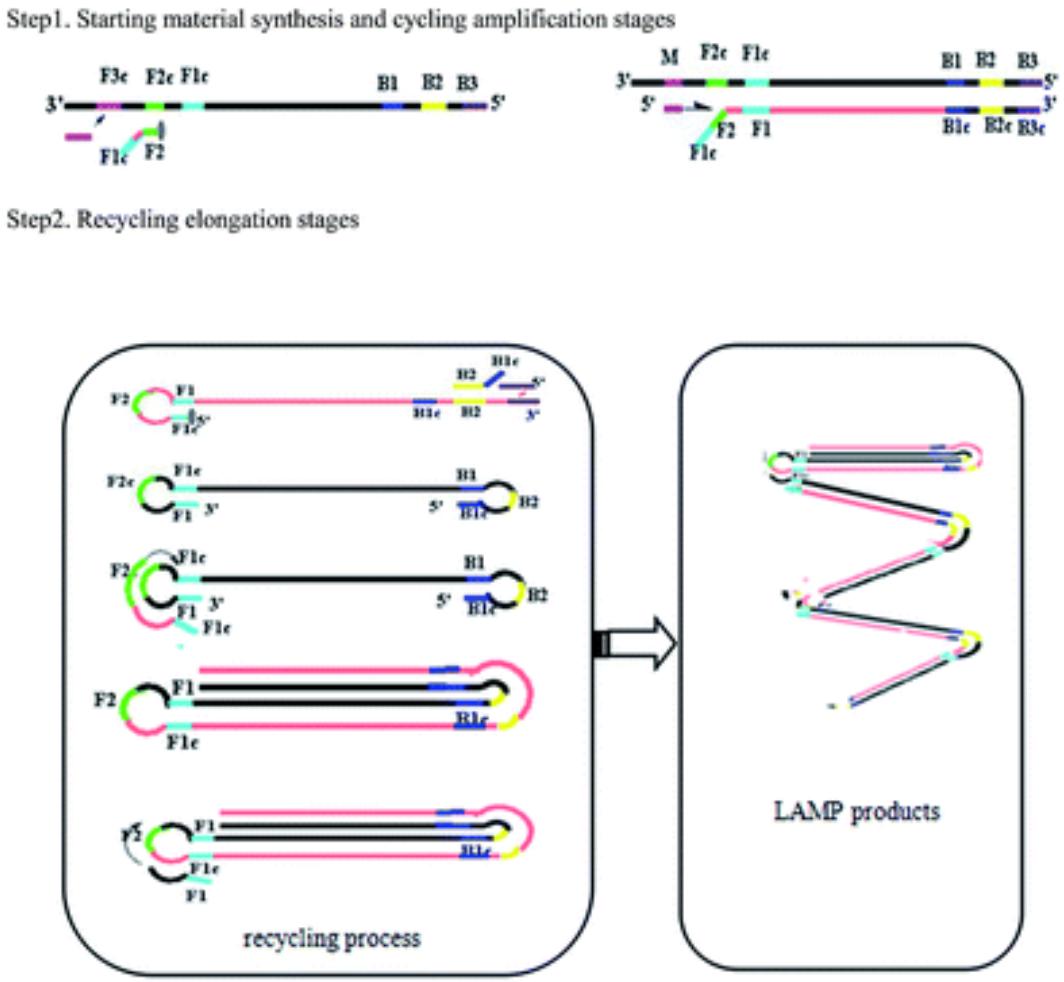

Figure 4: LAMP amplification process. Step 1 demonstrates the material synthesis and cycling amplification stages. A set of primers recognises six distinct regions at the target DNA and Bst DNA polymerase hybridise the starting material. The synthesised single-stranded DNA is displaced by the new strand of DNA which is synthesised by an outer primer. This acts as a template for the second inner primers. Step 2 demonstrates the recycling elongation stages. One inner primer synthesises at the loop-stem structure, this initiates the replacement of LAMP and produces up to 10^9 copies of the target gene in less than 60 minutes [63].

LAMP has proven to be a very popular isothermal amplification method for pathogen detection as it does not include a DNA denaturation step which is required when using PCR and real-time PCR based methods. It is also a very attractive method for point-of-use testing as it provides very rapid reaction times and is carried out using very simplistic operational procedures.

Gel electrophoresis is used as a method of validating DNA amplification. This method separates and analyses DNA based on its charge and size. Gel electrophoresis is commonly used in molecular biology and biochemistry to separate DNA and RNA fragments by length, to separate proteins by charge or to estimate DNA and RNA fragment size [64]. This is a simple method for DNA separation. Agarose gel in the form of a slab is used as the platform upon which small DNA molecules are “sieved” in a size-dependent manner. This is carried out by applying an electrical current across the slab of agarose gel. This works well for small DNA molecules and separation often fails where large DNA molecules are concerned [65].

SYBR green is widely used as a nucleic acid stain in molecular biology for methods including gel electrophoresis, real-time PCR and double-stranded DNA quantification.

However there are some challenges associated with LAMP as there are with any amplification method. Its high sensitivity can leave it susceptible to false-negative results due to issues of environmental contaminants [63].

LAMP has been used in various applications including pathogens which cause food-borne diseases such as Salmonella and E. coli. LAMP kits have been developed to detect such food-borne pathogens in an effort to reduce the time to detection associated with traditional culture based methods and reducing the laboratory workload [66].

LAMP is becoming an invaluable diagnostic tool in resource-poor countries where countless infectious diseases are affecting whole populations. The development of point-of-care (POC) devices for on-site quantitative screening of pathogens uses miniaturisation and combination of sample processing for cost-efficient sample-to-answer devices [63]. The integration of LAMP on POC devices is one of the future prospects for developing diagnostic tools that can be deployed in such resource poor settings to attempt to eradicate and control infectious diseases in affected developing countries [67].

Recently, Howson *et al.* has presented work where the detection of foot-and-mouth disease virus and HIV-1 viral RNA were achieved by employing two step reverse transcriptase LAMP assay [68]. This further cements the development of this amplification method as a highly credible tool in pathogen detection.

1.4.4 Nucleic Acid Based Amplification (NASBA)

Nucleic acid based amplification (NASBA) is emerging as a highly sensitive alternative to traditional PCR methods. This nucleic acid amplification method is a highly sensitive transcription-based amplification system specifically designed to detect RNA targets. The amplification reaction takes place at a temperature of 41 ˚C. There are three enzymes involved in this isothermal reaction: avian myeloblastosis virus reverse transcriptase, RNase H and T7 DNA dependent RNA polymerase (DdRp). This method is ideally suited for the amplification of RNA analytes such as mRNA and rRNA as there is the integration of reverse transcriptase into the system.

One major advantage of NASBA is the ability to produce a single-stranded RNA amplicon which can be directly used in another round of amplification. This single-stranded RNA can also be detected without requiring denaturation or strand separation steps. Another advantage of this method is that it is highly specific for RNA targets. In the presence of genomic DNA, mRNA can be specifically amplified and quantified without any influence of the corresponding genomic sequence. Although this is an isothermal reaction at 41 ˚C an initial melting step at 65 ˚C is required for the amplification reaction to proceed. This is a drawback of NASBA that can add additional time to the amplification reaction [69].

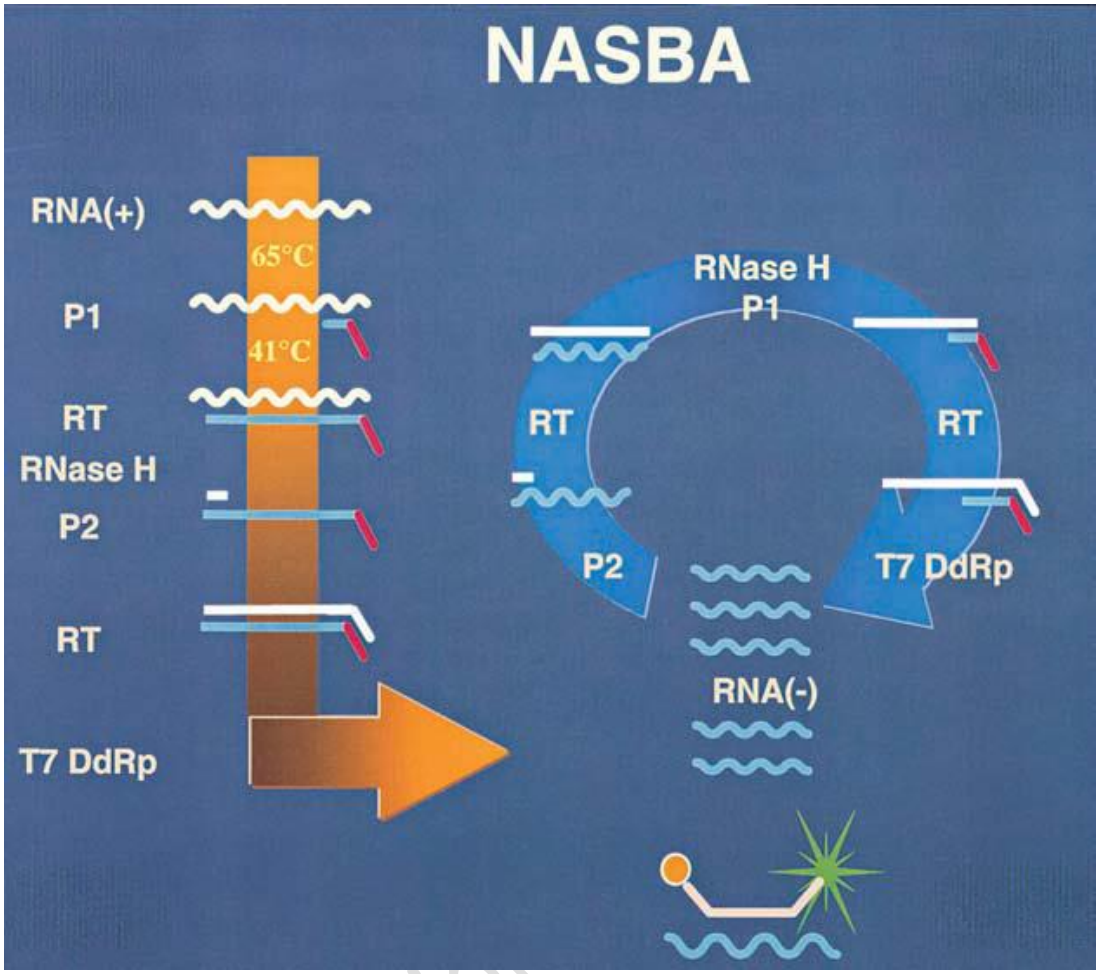

Figure 5: NASBA amplification process. This process includes molecular beacon detection. The initiation phase is represented by the orange arrow and the cyclic phase is represented by the blue arrow. Reverse transcriptase, RNase H and T7 DNA dependent RNA polymerase activities are all indicated. In the amplification phase, the polarity of the T7 DdRp product is complementary to that of the target nucleic acid [69].

1.5 Limitations of Screening

While genotype screening is extremely useful in a number of industries for a number of applications, there are a number of barriers which limit its economic impact. In the general case, genotyping requires the testing of a wide number of samples against a wide number of targets. This combination of M samples versus N targets implies the need to make major investments in laboratory infrastructure such as liquid handling robotics, thermal cyclers and measurement equipment. These approaches can require a vast number of repetitive pipetting steps where human factors introduce the risk of error into a system. For example,

screening one hundred samples against one hundred reagents, using PCR in triplicate, will require ninety thousand pipetting steps if performed by an operator or liquid handling robot using conventional methods. The large volumes associated with benchtop methods also make this an extremely costly process.

To attempt to address this issue there have been many attempts towards microfluidic large-scale integration. Thorsen *et al.* developed a high-density microfluidic chip which contains networks with thousands of micromechanical valves and hundreds of individually addressable chambers using soft lithography techniques. However they faced challenges regarding limitations from the intrinsic properties of the materials used to fabricate the devices. Another limitation was not all organic solvents were compatible with the materials used with soft lithography techniques. Contamination was also likely as a result of diffusion of small molecules through the elastomer used in the soft lithography techniques [70]. In this project the centrifugal microfluidic platform was identified as a suitable technology to advance towards screening applications.

1.6 Centrifugal Microfluidics

There are many advantages microfluidics can offer for applications in diagnostics and biological analysis. These advantages include small volumes, shorter time scales and high throughput of samples. Microfluidic devices can be used in a wide range of applications: nucleic acid microarrays; analyte detection; whole blood processing; detection of biochemical markers; cell and organism-based applications; cell-based assays for drug discovery; bacterial viability assay; capture and separation of cells [71]. Digital PCR on centrifugal microfluidic platforms is a major development in microfluidics. One of the main advantages of such a device is the detection of DNA mutations in DNA samples [72].

There is an increasing demand for *in situ* real-time measurements in the area of environmental monitoring. Microfluidic devices can provide a portable, robust and accurate way to carry out analysis of environmental samples in the field, and real-time measurement is required for autonomous, self-sufficient and robust methods of analysis to produce quality data. Microfluidic devices can accommodate such requirements with the integration of technology such as a low powered Wireless Integrated Network Sensors (WINSs) which can produce a network of autonomous "stations". An example of a microfluidic chip being used for environmental chemistry was developed using PMMA to determine two inorganic selenium species simultaneously as described by Madou *et al.* [73].

1.6.1 DNA Amplification on a Centrifugal Platform

An area that has developed greatly is in microfluidics is the ability to amplify DNA on a centrifugal platform. The advancement in this area is particularly useful for on-site applications such as testing for harmful food pathogens, diagnosis of harmful diseases and where GMOs are concerned. Commonly, DNA amplification is carried out using bulky, expensive instruments which require extensive training to operate. Centrifugal microfluidic platforms are increasingly being developed to be deployed on-site where they can be highly automated, user-friendly and very low in cost.

Madou *et al.* demonstrated a novel centrifugal microfluidic system where rapid amplification of a Bacillus anthracis gene was carried out by integrating pumping and thermoelectric-based heating and valving using PCR as the amplification method. This system demonstrated the first utilisation of ice-valving in an integrated centrifugal microfluidic system to efficiently seal the thermodynamic chamber to reduce the possibility of loss of sample due to evaporation. The fluidic thermal profiles were optimised to reduce the instance of non-specific amplification thus reducing the amplification time from 110 minutes to 53 minutes [74]. Focke *et al.* demonstrated a novel process flow enabling prototyping of microfluidic cartridges which were made from polymer films. This implemented a microfluidic genotyping assay which tested for twenty-two DNA samples from patients infected by methicillin-resistant Staphylococcus aureus (MRSA). The microfluidic discs were manufactured by soft lithography which applied positive moulds to allow higher moulding precision and simple demoulding compared to other conventional soft lithography techniques. This work demonstrated fabrication characterisation to allow the application of microfluidic cartridges with wall thicknesses of <188 µm which enabled reliable thermocycling during real-time PCR [75]. Quyen *et al.* used a device to significantly

reduce the detection time by utilising LAMP as a detection method for a common food-borne pathogen. This consisted of an injection moulded eight-chamber chip in combination with an optical reader for the rapid detection of *Campylobacter spp* which was obtained directly from pig at slaughter enrichment samples. PCR amplification was carried out by utilising an external heating element which was mounted under the microchip and plastic frame which contained eight magnetic elements clamped on top of the chips. A simple light transmission optical setup was used to efficiently read the PCR products. This significantly reduced detection time from five days to 30 minutes [76]. Tourlousse *et al.* developed a disposable polymer microfluidic chip which contained an array of fifteen interconnected reactions wells with on-board dehydrated primers for LAMP. They achieved a nucleic acid amplification chip allowing parallel detection of multiple pathogens in a low-cost and user-friendly platform. Hydrophobic air vents and microvalves were monolithically integrated into the multi-layered structure of the chip by utilising a cost-effective knife plotter to improve the loading and amplification on disc. Fluorescent DNA binding dyes were used to perform LAMP and the reactions were observed in real-time by a cost-efficient fluorescence imaging system which was previously developed within the group. However, only one single sample is tested for a number of different pathogens [77]. Park *et al.* demonstrated a centrifugal microfluidic device enabling multiplex foodborne pathogen identification by LAMP and colorimetric detection by utilising Eriochrome Black T. The analysis of twenty-five pathogen samples were carried out in five identical structured designed and integrated into the microfluidic system. The microchannels were zigzag-shaped allowing for optimum sequential loading and aliquoting of the primer mixtures, LAMP cocktail and the DNA sample solutions. This entire process was carried out in 60 minutes [15].

These are just a few examples of the advancements made in centrifugal microfluidics. However, there remain challenges around the automation of sample preparation and this

area can be significantly developed. There is also a need to develop centrifugal microfluidic devices which test a number of different sample types against a library of active reagents or pathogens allowing for a high level of pathogen detection in the food and agricultural industry.

1.7 Thesis Outline

This project was initiated to develop a sample-to-answer microfluidic cartridge for bacterial identification and serotyping from food samples. As part of this work, focus was placed on spatial multiplexing samples to identify bacteria serotype. In this approach a DNA sample is metered and aliquoted into multiple wells and each aliquot is then screened against a single LAMP primer set. This approach greatly reduces the complexity of LAMP assay design (as biological multiplexing is not required) and also the complexity of the operating instrument as the fluorescent detection system needs to operate at only a single wavelength and thermal cycling is not required.

As a natural extension of this screening of a single sample, this project was expanded to determine if a similar microfluidic cartridge can be used for MxN screening of multiple samples against multiple targets in an autonomous manner. With successful implementation, this approach might greatly reduce the cost and workload associated with all aspects of screening ranging from low-throughput point-of-use tests (akin to those which inspired this work) to massive throughput centralised analysis laboratories.

As this system is based on the centrifugal platform, Chapter 2 will describe key enabling valving technology and the theory underpinning their function. The fabrication and manufacture technologies used in the production of the microfluidic discs, which includes the cleaning and assembly process, are fully described in Chapter 3. The materials used

throughout this work and the testing procedures are described in detail. Sample preparation and DNA amplification and detection methods used throughout this work are also included in this section.

As a first demonstration of highly scalable combinatorial mixing we used a microfluidic disc to array two DNA samples with three primers (2x3). This is described in detail in Chapter 4.

A highly scalable 6x4 combinatorial mixing disc demonstrating twenty-four unique outputs is described in Chapter 5. This disc demonstrates a more complex microfluidic channelling and valving system to allow a high level of combinatorial mixing.

A progression of the 6x4 combinatorial mixing disc is demonstrated in Chapter 6. This disc demonstrates 10x10 combinatorial mixing towards an output of one hundred unique sample/reagent mixtures.

Based upon the need for an automated, low-cost microfluidic system for genotyping and screening applications, this thesis describes the development of a novel, highly-scalable, combinatorial, mixing centrifugal microfluidic disc to automate the mixing of a number of samples (M) for a number of active agents (N).

Chapter 2

Application of Microfluidics to Screening

This chapter describes in detail the various forces experienced on a centrifugal platform and the various integrated valving systems used for fluid transportation and control. The theory underpinning the function of these valves is also described.

2.1 Centrifugal Microfluidics

Lab on a Chip (LoaC) microfluidics is the emerging area associated with controlling and manipulating small volumes of liquids, with the use of small channels tailored of dimensions of tens to hundreds of micrometres, to perform common laboratory steps in a low cost, reliable and autonomous manner. Microfluidics can be sub-divided into a number of platform types including continuous flow microchannel devices, droplet based devices, digital microfluidic devices and paper-based microfluidics devices. These platforms perform fluid manipulation operations such as separating and metering, and mixing [78].

One such platform which is of increasing interest for use in diagnostic testing is the centrifugal microfluidic platform, or Lab on a Disc (LoaD). Centrifugal microfluidics pumps liquid primarily using the centrifugal force. Typically, a disc shaped cartridge of similar dimensions to a CD or DVD, is rotated about its central axis [79]. Liquid is pumped from the

centre to the edges of the disc and, using valves, individual Laboratory Unit Operations (LUOs) such as mixing, metering, washing, and aliquoting are performed.

The LoaD has a number of particular advantages for point-of-use applications. The system can perform centrifugation based sample preparation. In addition, just a spindle motor is required for pumping liquids; no external pumps are needed. Similarly, the chip can be loaded at atmospheric pressure and does not need to be sealed and pressurised for operation. These advantages are further described in Section 1.6.1. Along with the centrifugal force, the Coriolis and Euler force can also manipulate particles and liquids on the disc. These three forces possess different characteristics for fluid movement on disc.

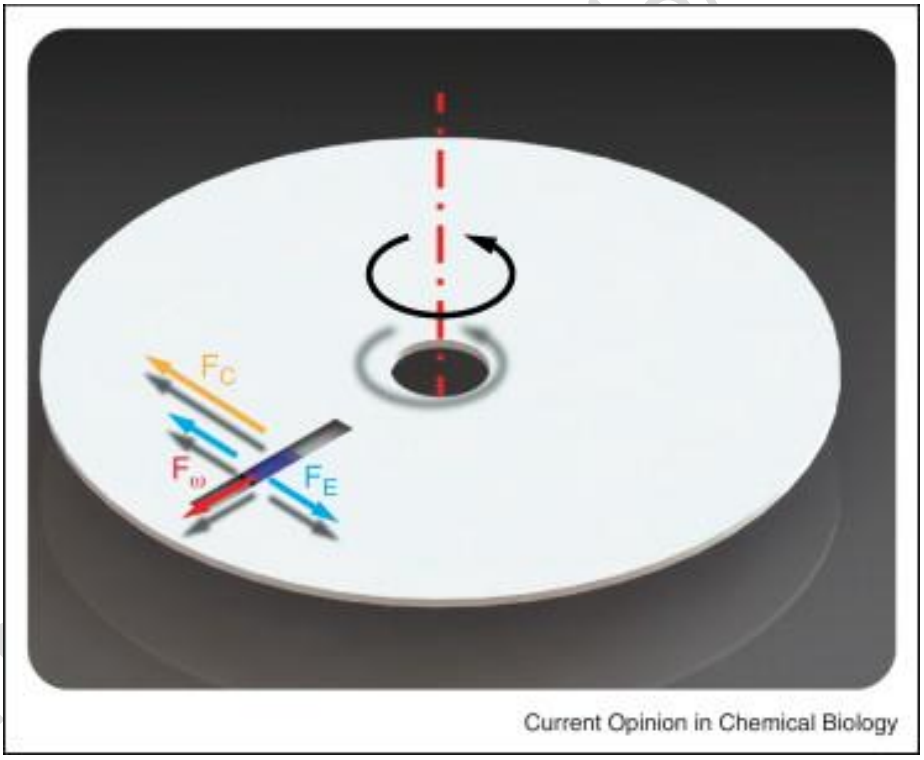

Figure 6: The forces experienced on a centrifugal platform affecting flow. F_ω is the centrifugal force, F_E is the Euler force and F_c is the Coriolis force. Adapted from Burger et al. [80].

The centrifugal force (density), F_ω, is the main acting force involved in centrifugal microfluidics

$$F_\omega = \rho r \omega^2 \qquad (2.1)$$

where ρ is the mass density of a fluid, r is the radial position from a central axis and ω is the angular velocity of the disc. This force propels fluids outward radially from the centre of the disc towards the disc periphery.

Another force experienced is the Euler force (density), F_E,

$$F_E = \rho r \frac{d\omega}{dt} \qquad (2.2)$$

where $d\omega/dt$ represents the rotational acceleration. Mixing of liquid is created by the Euler force allowing sample homogenisation. As the angular force changes with respect to time the Euler force is generated. This force acts in the opposite direction of the angular acceleration and is perpendicular to the centrifugal force.

A less influential force is the Coriolis force (density),f_c,

$$F_c = 2\rho\omega v \qquad (2.3)$$

where v is the fluid (or particle) velocity which is moving about the disc. The Coriolis force acts perpendicular to the centrifugal force. This can be applied in areas where flow switching techniques are required and also for particle separation [81]. While it can be applied for these applications, the Coriolis force is typically much smaller in magnitude and influence relative to the centrifugal force and thus its influence can often be disregarded.

Centrifugal microfluidic devices hold many advantages. The technology is based on a simple concept. A microfluidic disc, containing microstructures, spins about its axis using a low cost, compact spindle motor. This allows the propulsion of fluids from the centre of the disc radially outwards to the disc periphery due to the centrifugal force. As a result of this no external pumps are required to drive the fluid flow [82] and so there are no issue around 'world to chip' sealing of the microfluidic chip.

The microfluidic discs are typically manufactured from inexpensive polymer materials and so are very cost effective. As they are typically single use and disposable, they lend themselves well to human diagnostics and point-of-care / point-of-use applications. Centrifugal microfluidics is also a highly useful tool in biomedical diagnostics. The miniaturisation of processing steps such as mixing, metering, fluidic analysis can all be incorporated into these devices. As a result of multiple analysis steps easily integrated onto a single device multiple assays can run simultaneously. This contributes hugely in the development of microfluidic sample to answer systems [71].

Discs are typically mass-produced through plastic injection moulding or hot-embossing and are therefore low cost. Small batches for research purposes can also be manufactured using milling or the multi-layer methods described later in this thesis. Soft lithography is also useful for some research purposes, particularly for cell handing. This uses an elastomeric material such as polydimethylsiloxane (PDMS) to fabricate microfluidic devices by embedding surface patterns onto the soft material. A silicon master is used for producing the PDMS microchannels. The use of low cost materials is beneficial for device production as this results in the ability to mass produce devices at a low cost and the devices can be disposed of in an economical manner [83].

2.2 Rotational Flow Control

Centrifugal microfluidic platforms also face challenges due to the ubiquitous action of the centrifugal field on fluids during rotation. Integration of LUOs therefore requires reliable and controllable valves to control fluid movement about the discs. Valves are particularly important for isolating different liquids on disc from the rest of the system. This is useful for lysis and mixing of various samples and reagents. Accurate aliquoting is enabled by the use of valves to ensure a consistent metered volume of liquids. During assay processing valves are of high importance to control the fluid movement throughout the various channels and chambers. Valving techniques can be broken down into two groups: passive and active valves [79].

2.2.1 Passive Valving

In passive valving no other forces are experienced except for the forces present on the spinning disc. The rotation of the disc actuates the passive valves by increasing or decreasing the forces which depend on the centrifugal force (the apparent weight of a liquid) relative to the forces that don't change such as the capillary force. Examples of passive valves include capillary valves, hydrophobic valves and siphoning. Primarily these valves are controlled by the rotation of the disc [79]. Passive valves are typically open channels and so they do not seal the disc (and thus control the movement of vapour within the microfluidic device). Thus they cannot be used for on-disc storage over long periods of time [84].

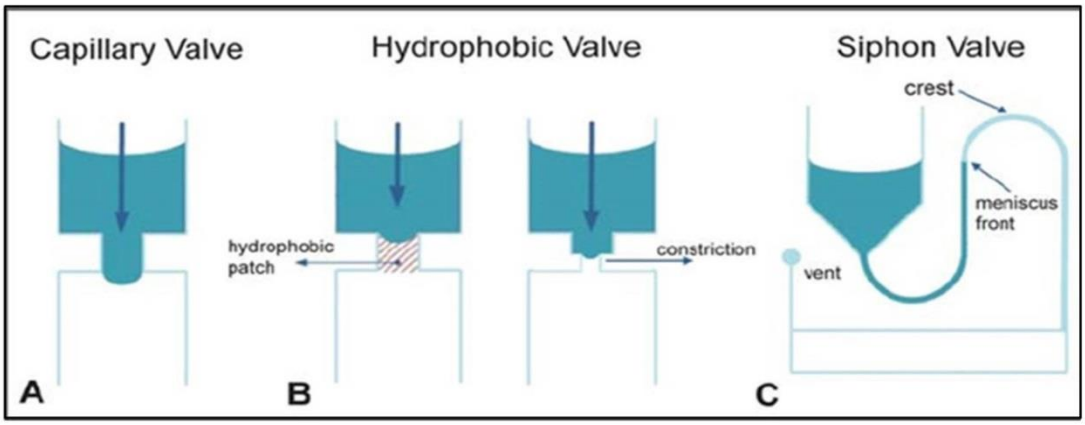

Figure 7: Valving systems integrated into microfluidic platforms as adapted from Gorkin et al. A) Capillary Valve with a hydrophilic microchannel, B) Hydrophobic Valve with the implementation of a hydrophobic patch, C) Siphon Valve showing fluid flow stopping at the siphon crest [71].

2.2.1.1 Capillary and Hydrophobic Valves

One of the most commonly used passive valves is capillary valves (*Figure 7* (A)). This type of passive valve is highly dependent on the balance between the centrifugal force and the surface tension force between the fluid and the disc material [84]. When a balance between the centrifugally induced pressure and the capillary pressure is present, the liquid will not move. If the disc rotation speed is increased, the centrifugally induced pressure will increase and overcome the capillary pressure and the liquid will be pumped through the valve. Capillary action will occur if, for example, a fluid is pumped from a small microchannel to a larger chamber; here a surface tension force will be created and will stop (or slow) fluid movement. In order for the capillary valve to open the centrifugal pressure must be greater than the capillary pressure. This can be achieved by increasing the rotational frequency of the disc which results in an increase in the capillary pressure. The disc spin rate at which this occurs is known as the burst frequency ω_c. The surface tension is overcome and the fluid will continue to flow in the system.

Although capillary valves are commonly used throughout centrifugal devices there are some limitations involved with this type of passive valve. In some cases the surfaces must be hydrophobic while in other variations surfaces are required to be hydrophilic. These requirements mean often additional surface treatment steps need to be carried out during manufacture. The valve performances are highly dependent on contact angle and this can vary over time if discs are not stored under optimum conditions. The burst frequency is also highly dependent on the minimum channel geometries and the fidelity of disc manufacture (including surface roughness and edge definition). These mean a trade-off between the cost of manufacture and the performance of the valves. Manufacturing limitations place a maximum spin-rate on these valves, above which the valves will almost always open, and also results in 'smearing' of the valve burst frequencies; therefore each valve must be designed to open at a frequency well-spaced from the next one on the disc [85].

Hydrophobic valves are a very simple but effective type of passive valve. A small hydrophobic patch is used for stopping fluid flow. This hydrophobic patch is placed along the channel as shown in Figure 7(B). Hydrophobic valves can be applied in two different ways. The first obstructs the movement of fluid by situating a hydrophobic patch in functional areas of the disc. The second can be carried out by narrowing a hydrophobic channel [71]. Limitations also arise with this type of passive valve. Hydrophobic valves cannot withstand high rotational frequencies and therefore burst at relatively low frequencies similar to capillary valves [85].

2.2.1.2 Siphon Valves

The siphon structure (Figure 7(C)) is widely used as a valving mechanism. This type of valve can withstand higher rotational speeds in comparison to capillary valves [79] and is in fact a low-pass valve; it opens with a reduction in the spin frequency. The siphon channel is primed by capillary action and so for this to occur the siphon channel must be hydrophilic [71].

Figure 7(C) includes a siphon channel structure which is connected to a chamber filled with fluid. The siphon channel extends higher than the radial position of this chamber. The siphon channel is also connected to another chamber that is situated at a lower radial position [85]. The centrifugal force results in the meniscus of the fluid are kept below the crest level of the siphon when high rotational frequencies are applied to the system. This ensures that the fluid is maintained at a constant level [71]. When the rotational frequency is decreased the centrifugal force weakens and so the fluid is transferred along the siphon channel due to capillary action. The fluid is transported to the chamber positioned at a lower radial position and primes the siphon channel [85]. Following the priming of the siphon the rotational frequency is then increased again to allow the first chamber to completely empty along the siphon channel and into the second chamber. The siphon valve structure is particularly useful when initial high rotational frequencies are required on disc. However, the siphon channel requires priming by capillary action and therefore the siphon channel must also be hydrophilic. There is also a limitation with this type of passive valve as often a lot of surface area is required to obtain a highly optimised siphon structure [71].

2.2.1.3 Dissolvable Film Valves

The primary valves used in this project are derived from dissolvable films (DF). The first DF valve technologies, Burst Valves, were first introduced by Gorkin *et al.* and can be categorised as a passive valve. This work demonstrated a novel valving system that could be integrated on a centrifugal microfluidic platform to perform fluid manipulation [82].

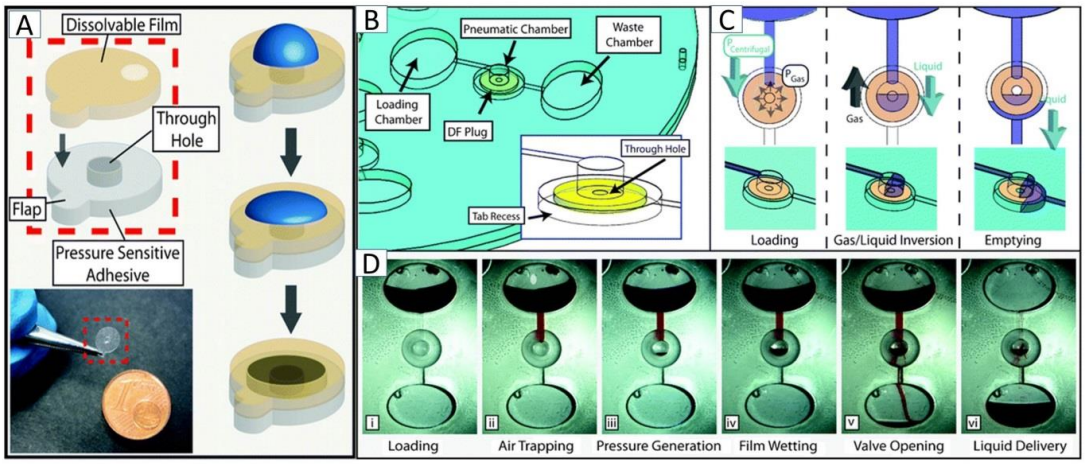

Figure 8: Centrifugo-Pneumatic DF valving. A) Shows the structural design and scale of a DF valve. The film starts to deteriorate upon contact with liquid and eventually completely dissolves. The fluid moves through the remaining structure B) Demonstrates the centrifugo-pneumatic valving concept. This shows the integration of a DF valve into a microfluidic system. C) Shows the DF valve in contact with fluid in a microfluidic system under centrifugal conditions. This demonstrates the operation at the point of loading, during gas/liquid inversion and after film dissolution and emptying of the sample fluid D) Food dyes are used to demonstrate the DF valve operation. At low spin speeds the air is trapped, air is compressed at increased rotational frequencies resulting in air inversion in the chamber. Liquid introduced into the valve results in the disintegration of the DF and valve opens. Adapted from Gorkin et al. 2012 [82].

In this system Figure 8 (B), the DF valve is placed in a pneumatic chamber between the loading chamber and the waste chamber. This creates a pocket of trapped air in the microfluidic disc. At low rotational speeds the fluid present in the loading chamber is unable to wet the DF due to the trapped air. When the rotational speed of the disc is increased the fluid in the loading chamber can overcome the pressure created by the

trapped air and is allowed to flow into the pneumatic chamber. This results in the wetting of the DF valve. After the fluid comes into contact with the DF valve the valve then dissolves and the fluid flows into the next chamber.

The controlled movement of fluids can be optimised and achieved by using this DF valve as a fluid barrier. One of the major advantages of using DF valves is the ability of select DFs which take different times to dissolve in water. The integration of DF valves with varying dissolution times creates a more automatable system and adds more control to the valving process [82].

Further tuning of the valve can be carried out by changing the size of the pneumatic chamber. Larger chambers require a lower burst frequency to permit the liquid to come in contact with the DF.

The valving mechanism outlined here is highly dependent on the balance between the pressure head ΔP and a critical yield pressure P_{crit}. These pressures are centrifugally induced in this system and are derived from the standard equation for hydrostatic pressure,

$$\Delta P = \rho g h \qquad (2.4)$$

where g is the angular acceleration and h is equal to Δr, for the rotating frame.

As the centrifugal acceleration acting on the liquid increases with radial location, equation (above) can be rewritten as

$$\Delta P dr = \rho \omega^2 r dr \qquad (2.5)$$

integrating

$$\int \Delta P dr = \rho \omega^2 \int r dr \qquad (2.6)$$

applying the bounds of the liquid element

$$\int \Delta P dr = \rho\omega^2 \int_{r_0}^{r_1} r dr \qquad (2.7)$$

integrating

$$\Delta P = \rho\omega^2 \left[\frac{r^2}{2}\right]_{r_0}^{r_1} \qquad (2.8)$$

applying the limits

$$\Delta P = \rho\omega^2 \left[\frac{{r_1}^2 - {r_0}^2}{2}\right] \qquad (2.9)$$

expanding

$$\Delta P = \rho\omega^2 (r_1 - r_0)\left(\frac{r_1 - r_0}{2}\right) \qquad (2.10)$$

Rewriting in commonly used terminology in centrifugal microfluidics where $\bar{r} = [((r_0 + r_1)/2]$ and where $\Delta r = (r_1 - r_0)$

$$\Delta P_\omega = \rho \Delta r \bar{r} \omega^2 \qquad (2.11)$$

The density is represented by ρ , Δr is the radial length, $\bar{r}$ is the mean position of the liquid plug and ω is the angular frequency. Absolute pressure is measured by

$$P_{abs} = \Delta P_\omega = \rho \Delta r \bar{r} \omega^2 + P_{atm} \qquad (2.12)$$

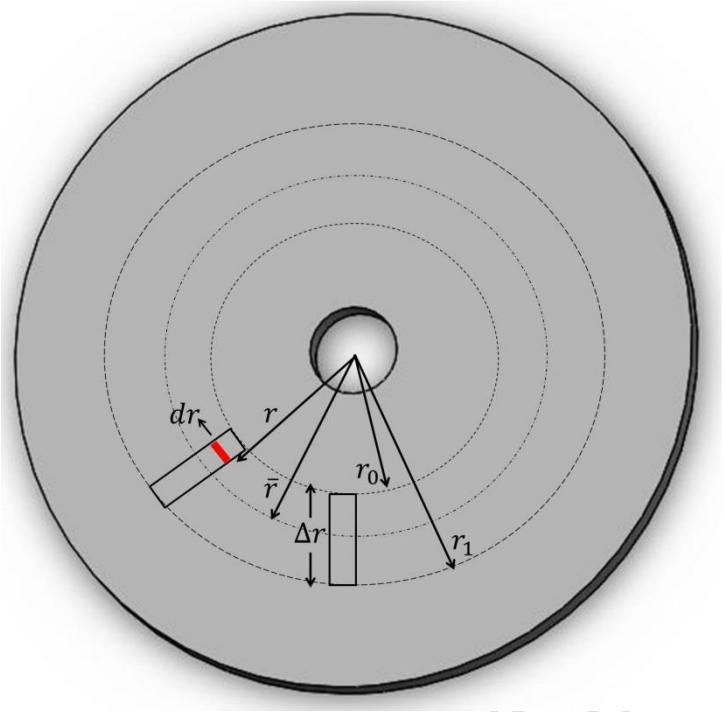

Figure 9: Definition of the position of the fluid plug on a centrifugal system. r_0 is the radially inwards point of the fluidic plug and r_1 is the radially outward location of the fluid plug. To calculate the centrifugally induced hydraulic pressure at location r_1, the key dimensions are Δr, which is the radial length of the fluid element [(r_1-r_0)], and $\bar{r}$, which is the average radial position of the fluid plug [((r_0+r_1)/2]. dr is an infinitesimal element and r is it's distance from the centre.

The inner point of the fluid element is given by r_0, and the outer point of the fluid element is given by r_1.

The liquid meniscus stops as ΔP_ω is balanced by pressure in the valve chamber

$$P = P_{atm} \frac{1}{1-\frac{\Delta V}{V_0}} \qquad (2.13)$$

This is derived from Boyle's law.

The equilibrium system can be modelled by equating the pressure differences

$$P_{atm}\left(\frac{1}{1-\frac{\Delta V}{V}}-1\right)=\rho\Delta r\bar{r}\omega^2 \qquad (2.14)$$

This can be rearranged for ω to determine the burst frequency

$$\omega=\sqrt{\frac{P_{atm}\left(\frac{1}{1-\frac{\Delta V}{V}}-1\right)}{\rho\Delta r\bar{r}}} \qquad (2.15)$$

where, ω, is the angular velocity in rad/s and the burst frequency, Hz, is equal to $\omega/2\pi$..

In the original geometry presented by Gorkin *et al.*, surface tension helps stabilise the liquid-gas interface. The liquid head moves further through the pneumatic chamber as the centrifugal pressure ΔP_ω head increases. This makes it harder for the surface tension to sustain the "hanging" liquid volume ΔV. The liquid plug disrupts to invert the liquid-gas configuration at the burst frequency. The DF dissolves and opens the valve as the liquid proceeds into the pneumatic chamber [86]. Thus, in this geometry, the valve has a low dead volume but also a degree of unreliability as it is dependent on the often unpredictable surface tension.

In advancement, Dimov *et al*. introduced an upturned channel where the heavy liquid is positioned below the lighter gas. This resulted in surface tension having less impact on the valve performance and, with the caveat that the valve has an increased dead volume, improved reliability and agreement with theoretical models [87]. This valving geometry is used in the experimental section of this project and is shown in greater detail in Section 3.2.2.

Another type of DF valving is the event-triggered valves. These valves cannot easily be defined as active or passive. Here, valve actuation is based on movement of liquid about

the disc rather than changes in disc spin rate or frequency. This advancement is based on a fluid flow triggered by the release of another fluid on a different location on the disc. This introduces a liquid handling sequence which can be controlled by a response to completion of a preceding liquid transfer located separately on the disc. This network of valving allows for large-scale process integration. One of the major advantages of using event-triggered valves is the independent nature of the process from the rotational frequency of the disc [88].

2.2.2 Active Valves

Active valves have a greater reliability and robustness in comparison to passive valves. This is due to active valves requiring an external actuation mechanism. Unlike passive valves, these valves are independent of the angular velocity of the disc. Sacrificial materials are typically used for active valving in microfluidic systems. These sacrificial valves act as a barrier in the channels and can be situated throughout the disc to isolate fluids until time of need [84] as a reagent storage mechanism. These barriers can be disrupted by the application of physical or chemical stimulants allowing the fluid flow to continue [82]. Typically, active valves will be a paraffin wax which has been embedded with ferrous nanoparticles. Heated by an infrared laser, the wax will melt and be displaced out of the channel to permit a liquid to pass. Some of these valves can be re-heated to close a channel after the liquid has passed.

Chapter 3

Materials & Methods

This Chapter describes the materials and methods used throughout this work. The experimental work is categorised into four stages: fabrication of parts, biological sample preparation, DNA amplification and testing. Specific manufacturing relating to disc design is described in their relevant chapters.

3.1 Fabrication of Parts

Microfluidic discs are purposely designed and built to combine a network of multi-scale channels and chambers which manipulate fluid movement through a sequence of laboratory unit operations (LUOs). The multi-layer system is described in more detail in Chapter 3. There are 3 steps involved in the fabrication process of the microfluidic discs: design of the layers, fabrication of the parts, and the final assembly. Design of the discs was primarily carried out using SolidWorks (Dassault Systèmes) version 2014 and 2015.

3.1.1 Rationale of Materials used in Disc Manufacture

Initially silicon or glass substrates were used in the fabrication process of microfluidic discs due to the optical transparent property of glass. Over time these materials presented limitations due to their high cost and complicated manufacturing procedures which caused users to find alternative materials [89]. Polymers presented a more cost efficient alternative. Poly(methyl methacrylate) (PMMA), Poly(dimetylsiloxane) (PDMS), Poly(styrene) (PS) and Poly(carbonate) (PC) are widely used as a replacement due to their

low-cost, disposability, easy manipulation and have proven to create a less complicated fabrication process [90].

In microfluidics PMMA is widely used for the fabrication of devices due to its very low hydrophobic property. It is an ideal material for microfluidic devices as it is very cheap and can be easily disposed after a single use [89]. It is one of the most commonly used polymers in microfluidics due to its excellent electric; mechanical properties; its excellence in optic transparency and is a perfect candidate for the use of laser ablation to excellently laser cut out designs for fabrication, fast prototyping and production [91] . However PMMA has a rugged surface and a limited surface chemistry which is a limiting factor in microfabrication [92].

Other rapid prototyping materials such as PDMS by soft lithography and the use of polystyrene (PS) are not compatible with these requirements. The use of PDMS in the manufacture of microfluidic systems has been widely used for decades [93]. However, one of the limiting factors of using PDMS is its ability to absorb small hydrophobic molecules. PS is favourably used among biologists for cell-based studies as a ubiquitous material for tissue culture plastic ware. However, PS cannot be fabricated using $C0_2$ based laser cutter which is the manufacturing method of choice throughout this work. To this end hot-embossing and injection moulding cannot be used as other rapid prototyping techniques as they cannot provide the multi-layer architecture required for the manufacturing of these microfluidic discs [93]. PMMA was chosen as the material used in the fabrication of the microfluidic devices throughout this experimental work as it is highly compatible with available laser cutters. For these reasons the material used for the manufacture of discs throughout the course of this work was PMMA and for its compatibility with biological materials.

PSA (Pressure Sensitive Adhesive, Adhesives Research, Limerick, Ireland) is used to both hold the PMMA layers together and provide microfluidic channels between layers of the disc. This provides the multi-layer architecture which is a vital aspect for this work. This is a double-sided adhesive which adheres to surfaces when pressure is applied either by hand or by mechanical means [94]. PSA is a very strong adhesive material and cannot be re-used due to its strong attachment to a surface. Assembly was carried out under clean room conditions to ensure a clean surface between the PMMA and PSA for optimum bonding.

DFs were used in the manufacture of the microfluidic disc for fluidic control and reagent storage [86]. This is described in detail in Section 2.1.2.3. DFs are commonly used as fast dissolving oral films for drug delivery systems. DFs are used throughout this work as they are water soluble and are extremely thin materials making them an excellent material to integrate into microfluidic discs [95].

3.1.2 Fabrication Process

Laser ablation of PMMA was used throughout this work as it was effective in achieving rapid prototypes for microfluidic testing and a very cheap material to use. The PMMA layers of the disc were fabricated using a CO_2 based laser cutter. Large features were created in the PMMA layers by using laser ablation to create structures such as reservoirs and vertical vias allowing fluid movement throughout the disc.

PSA features were cut using a Graphtec knife cutter. Small structural features were created by cutting voids out of the PSA layers, allowing them to act as channels for fluidic transportation. The layering of the PMMA and PSA layers creates a three dimensional network of chambers and channels for flow control.

A multi-layer system is manufactured using dissolvable film creating a three dimensional internal structure. A valving system, between layers is created using dissolvable film pneumatic valves that are composed of DF supported PSA with a pneumatic chamber created by a void within the PSA. The movement of fluid is controlled with a high degree of accuracy due to this three dimensional internal structure.

The complete assembly was carried out on a specialised alignment stand with the use of alignment pins for precise manufacture. The discs were rolled with a hot roll laminator (Hot Roll Laminator, Chemsultant Int., US) for efficient, reproducible sealing of the layers throughout the assembly process. The complete fabrication processes are described in further detail throughout this chapter.

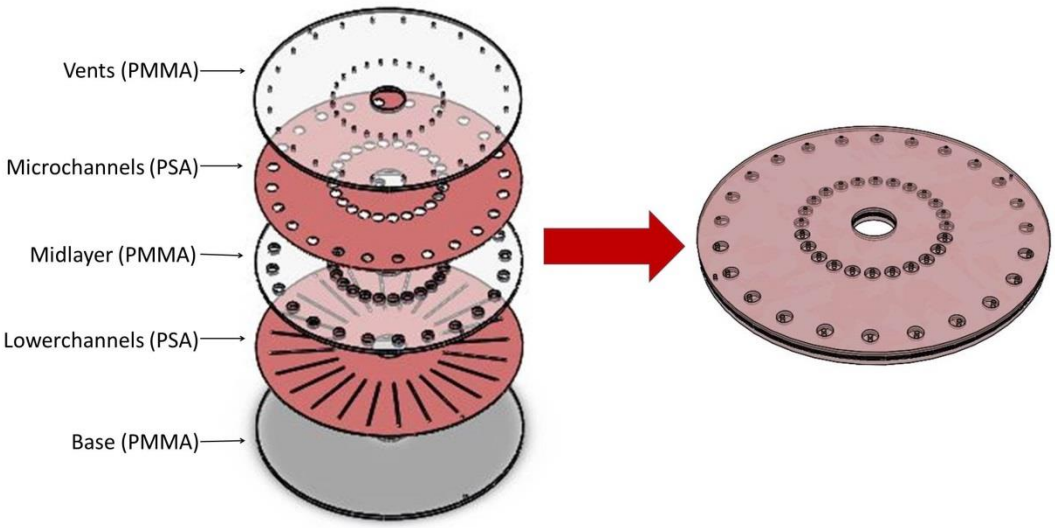

Figure 10: The disc assembly process without the integration of DF tabs. The PMMA layers are clear/light grey and the PSA layers are a light red colour. These layers are assembled under clean room conditions to produce the complete microfluidic disc.

3.1.2.1 Laser Machining

Laser ablation was used in the fabrication process of the PMMA layers. This method using laser ablation is a rapid technique commonly used in disc prototyping. A CO_2 laser is used to cut structures from the PMMA substrate. Designs are created using CAD software, such as SolidWorks, and designs uploaded onto the system in a .DXF file format for automatic machining [92]. The PMMA melts and vaporises upon contact with the focused CO_2 laser (wavelength of ≈10.6 μm) [96] . The design is cut out as the laser follows its instructed path resulting in the desired cut out design. Parameters can be adapted to achieve a wide range of cut depths which include the laser power, speed and number of passes the PMMA experiences from the laser. The thickness of the PMMA is also a contributing factor and also has an effect on the cutting behaviour of the PMMA [97]. Difficulties with the CO_2 laser cutting can arise as bulges form as a result of the rapid heating of the PMMA. Issues may also arise with the bonding of the PMMA layers in the assembly as a result of these bulges.

The Epilog Zing 16 (Epilog Laser, USA) was used for the duration of this work during the microfabrication process for laser cutting the PMMA. It has a material thickness of maximum 114 mm and the total working area is 406 x 305 mm with a digitally controlled air-cooled 30 W CO_2 laser feature.

The PMMA cutting parameters are given in Table 1.

PMMA cutting parameters		
PMMA thickness	Machine Speed (%)	Machine Power (%)
2 mm	18	40
1.5 mm	20	24
1 mm	25	20
0.3 mm	21	16

Table 1: Cutting parameters for various PMMA thicknesses

3.1.2.1.1 Laser Machining of PMMA

The desired thickness PMMA was removed from its covering material. The features were designed in Solidworks and transferred to the Coreldraw software as vector drawings. The Epilog Zing laser cutter was used to cut the PMMA with the appropriate parameters listed in Table 1.

3.1.2.1.2 Laser Machining of PMMA and PSA

The desired thickness PMMA was removed from its covering material. A piece of PSA was cut to the desired size and the clear protective layer was removed from one side of the PSA. The piece of PSA was laid flat on a working surface. The PMMA was placed on top of the PSA and pressure was applied to stick the PSA and PMMA material together. A hand held roller was used to remove any air bubbles created during this process. The features were designed in Solidworks and transferred to the Coreldraw software as vector drawings. . The

Epilog Zing laser cutter was used to cut the PMMA with the appropriate parameters listed in Table 1.

3.1.2.2 Knife Cutting (Xurography)

Xurography is the process of rapidly prototyping microstructures in adhesive films using a precision knife cutter. This process is widely used in the area of microfluidics for rapidly prototyping discs [98]. A motorised precision knife cutter was used to cut out the desired design on the PSA layers. The Graphtec Cutter Plotter CE6000-40 (MDP Supplies, Republic of Ireland) was used for the duration of the microfabrication process. Designs were prepared in Solidworks and transferred to Graphtec Studio for transmission to the knife cutter. PSA was cut to desired size and loaded onto a support sheet. The PSA was cut using cut force 21 at a machine speed of 2.

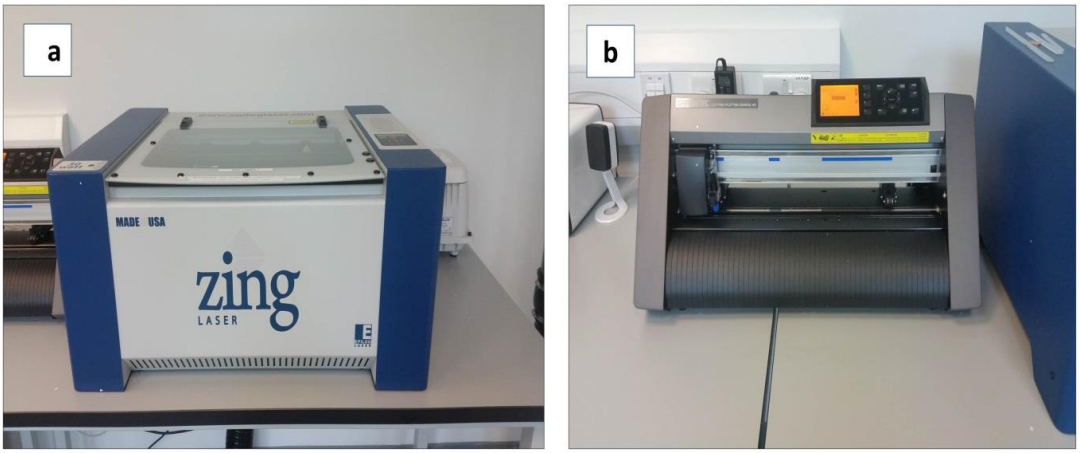

Figure 11: Equipment used in the microfabrication process. a) Laser cutter used to cut PMMA discs. b) Knife cutter used for Xurography process to cut small structures into the PSA layers of the disc.

3.1.2.3 Dissolvable Film Tabs

Dissolvable films (DF) were used in the assembly of the microfluidic discs for control of fluid flow. Adherent tabs were created, for the integration in the DF valving system, by placing a small piece of DF onto one sheet of PSA. This created a double sided encasing for the DFs as the films are non-adhesive [82].

The knife-cutter created through-holes in the PSA which allowed the flow of fluid once the DF had dissolved. The DF tabs are designed using CAD software by patterning equally spaced inner circles. A second sketch is created which defined the outer edge of the DF tabs. The knife-cutter was used to create the inner holes on the PSA and was manually removed using a tweezers. The clear protective layer on the PSA was removed, exposing the adherent surface, to allow the DF sheet to stick. The outer edges of the DFs were then cut creating the DF tabs. The cutting force used for the outer edges was decreased to ensure the DF tabs remained mounted on the backing film of the PSA for storage until disc assembly. This ensured the DF tabs could be readily peeled off the backing film during disc assembly.

Throughout this work three types of DFs were used FA-35 film and KC-35 film (Harke Packpro, Germany)(which will be referred to as FA and KC film respectively) and a DF low in cost typically used in the process of embroidery (Barnyarns, Ripon, UK; Avalon) which will be referred to as Embroidery film. The FA and KC DFs are manufactured from a water-soluble film which is based on the synthesis of polyvinyl alcohol (PVA). These DFs are made from SOLUBLON®. The Embroidery film had a composition of mostly PVA. The different DFs had varying dissolution times when they come into contact with DI water. The different DF dissolution times were tested and characterised as previously reported [99].

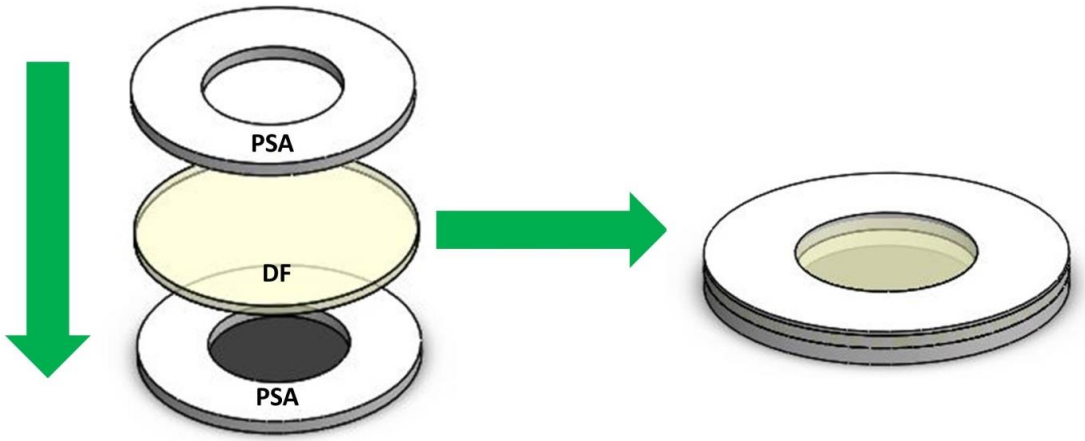

Figure 12: The manufacturing process of DF tabs. A DF is sandwiched between layers of PSA containing through-holes to allow fluid to pass through once the DF has dissolved.

The DF used in the manufacture of this disc varied between KC and embroidery. This was varied dependent on the preferred liquid release time as both of these films hold different dissolution times. KC has a dissolution time of 30 seconds and was used in the initial manufacture and microfluidic testing to allow increased spin time for design optimisation. After design optimisation and rigorous microfluidic testing using coloured food dyes, the DF tabs were manufactured using embroidery which has a dissolution time of 3 seconds.

3.1.3 Cleaning Procedure

An Ultrasonic bath (Grant Instruments, Cambridgeshire, UK) was used to clean the PMMA layers. This cleaning process removed any unwanted particulates that were attached to the disc as a result from the laser ablation. For biological testing this is of utmost importance to avoid any unwanted interactions with the biological sample.

3.1.3.1 MICRO90 Washed PMMA

First the PMMA layers were placed into a plastic bag with a 2 % (v/v) solution of MICRO90 in deionised (DI) water. The bag containing the 2 % MICRO90 solution and PMMA was heat sealed to ensure no loss of solution or contamination by the water of the sonic bath. The plastic bag was placed into the sonicator at 50 ˚C for a total of 30 minutes. After 30 minutes the bag was removed from the sonicator and the PMMA layers were thoroughly rinsed with DI water. The PMMA was then sealed into a fresh bag with DI water and placed into the sonicator for a further 30 minutes at 50 ˚C. The PMMA layers were then removed and given a final rinse with DI water. A nitrogen stream was used to blow dry the PMMA layers once thoroughly cleaned. Before assembly the PMMA layers were cleaned with a lint-free wipe and 70 % isopropanol [87].

3.1.3.2 MICRO90 Wash and BSA Blocked PMMA

The PMMA layers were washed according to Section 3.1.3.1. Following that cleaning procedure a 3 % bovine serum albumin (BSA) solution was prepared by dissolving BSA with nuclease-free water. The 3 % BSA solution was added to the bag, heat sealed and placed in the sonicator at 50 ˚C for a further 30 minutes. The PMMA was removed, placed on lint-free wipes and allowed to air dry.

3.1.4 Disc Assembly

The assembly process was performed under clean room conditions and required the equipment as shown in Figure 14. Transfer film was used as the backing support for the PSA layers which aided in the adherence to the PMMA layers. The complex structures created in the PMMA and PSA layers must be perfectly aligned to ensure the interconnection of the

multiple layers. The layers were aligned using the alignment rig and alignment pins. The alignment pins were carefully placed through the alignment holes in each PMMA and PSA layer to ensure correct alignment was achieved. A hot roll laminator (HL-100 Hot Roll Laminator, Cheminstruments, USA) was used to apply extremely high pressure to ensure efficient sealing of the PSA and PMMA layers. Each individual PSA and PMMA layer was passed through the roller a minimum of five times at a pressure setting of 80 psi throughout the assembly process to ensure maximum sealing efficiency was achieved. This is to prevent leaks occurring during testing.

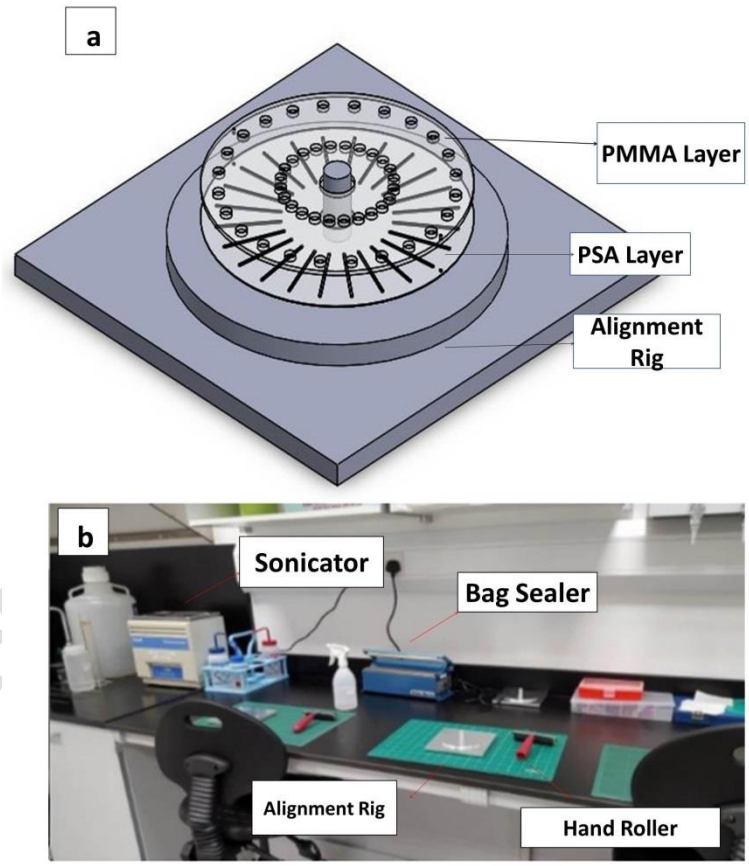

Figure 13: Equipment used for assembly in clean room. a) Schematic of the assembly rig with PMMA and PSA layers. b) Assembly room set up for disc preparation and assembly.

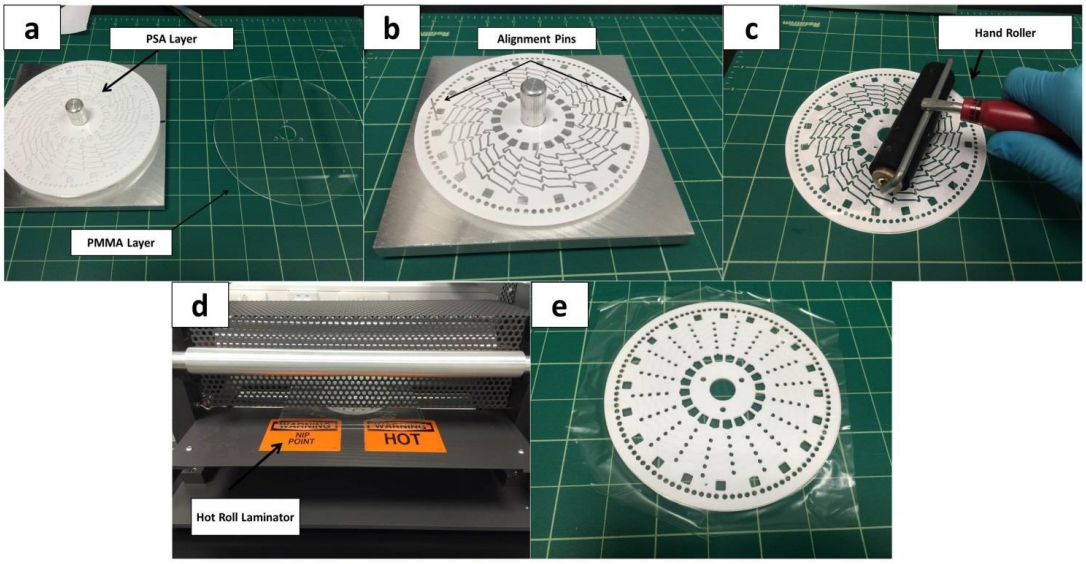

Figure 14: The disc assembly process under cleanroom conditions. a) The PSA layer mounted onto the alignment rig with the PMMA layer ready for assembly. b) The PSA and PMMA layers are aligned using the alignment pins. c) The two layers are manually rolled using a hand roller. d) The disc is then placed between the two rollers in the hot roll laminator and clamped shut. This is rolled a minimum of five times rotating the disc between each pass to ensure pressure has been applied to all areas. e) The complete assembled disc is placed in a heat sealed bag until testing.

A standard microfluidic disc consists of four PSA layers and four PMMA layers shown in Figure 15. Layer 1 consists of vent holes (PMMA) to allow air to enter/leave a chamber as fluid enters/leaves. This ensures that the chamber remains at atmospheric pressure. Layer 2 consists of higher-level microchannels which serve as channels for liquid transport and pneumatic venting (PSA). Usually these microchannels are 50 µm in width and have a height of 86 µm due to the thickness of the PSA. Layer 3 contains vertical vias in the form of reservoirs and through-holes (PMMA). These large structures permit mixing and liquid storage. Layer 4 is the top cover layer acting as a sandwiching layer to hold the DF tabs in place (PSA). Layer 5 is the bottom support layer which contains voids to hold the DF tabs in position (PSA). The DF tabs are part of the DF valving system which restricts the movement of fluid throughout the disc by creating a build-up of pressure as the fluid approaches the

valve. This pressure is overcome by increasing the rotation frequency of the disc. Layer 6 contains through-holes which act as vertical vias (PMMA). Layer 7 consists of the lower-level microchannels which guides fluid flow and pneumatic venting (PSA). Layer 8 acts as a base layer for mechanical backing. To enable and allow channel crossing, layers 2 and 7 are isolated from each other to permit this action to occur.

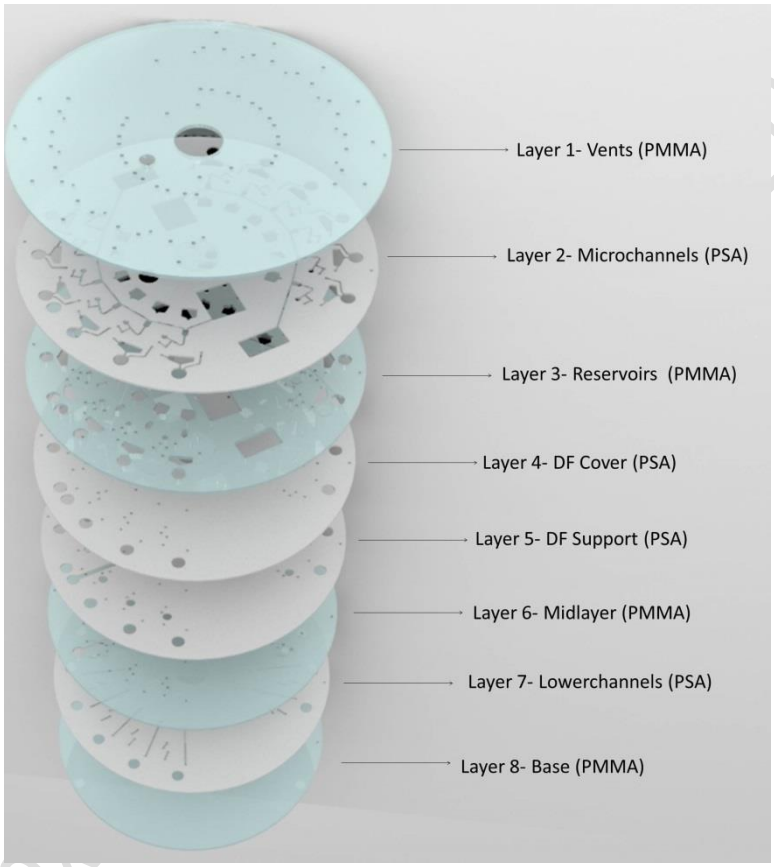

Figure 15: The assembly of a microfluidic disc with four PSA and four PMMA layers. The PMMA layers (light blue) and PSA layers (white) are assembled into a complete disc under clean room conditions.

3.1.4.1 Disc Assembly Using DF Tabs

The disc assembly using DF tabs is carried out as outlined in Section 3.1.4. During the disc assembly the DF tabs manufactured as outlined in Section 3.1.2.3 were carefully positioned to the DF tab voids between layers 4 and 5. The DF tabs were placed onto layer 5 with

precision using a tweezers. Layer 4 was placed on top to act as a sandwiching layer to hold the DF tabs in place. This was rolled through the hot roll laminator and assembly proceeded as outlined in the assembly procedure in Section 3.1.4.

3.1.4.2 Disc Assembly Using Whole Sheet of DF

A whole sheet of DF was used instead of the DF tabs. This sheet was cut to size and laid flat on the work bench during assembly. A disc with an exposed sticky surface of PSA was placed carefully on top of the DF sheet. This was rolled smooth with a hand held roller. A PSA layer was then placed on top of the exposed side of the DF sheet which formed a sandwiching layer holding the DF sheet in place. This was then placed in the hot roll laminator and assembly proceeded as outlined in Section 3.1.4.

3.2 Microfluidic Testing

A spin stand was used in the testing of each microfluidic disc as this generated the centrifugal force which was imperative throughout this work. A camera and strobe light are synchronised to a trigger signal generated once per motor revolution. This signal is transmitted to the strobe / camera and an image is acquired. As the disc is in the same angular position during acquisition of these frames, the disc appears stationary despite rotating at up to 70 Hz. Thus, fluid movement about the disc can be easily imaged. The layout of the experimental spin stand set up is shown in Figure 16. A spindle motor (CMMS-AS Servo Motor, Festo, Germany) was used as a support fixture for the microfluidic disc. The disc was fixed securely in place and spun at a low speed to optimise the spin stand parameters. The speed was controlled by a Labview programme designed in-house for the application of disc testing. The brightness and visibility was adjusted by adapting the height and position of the strobe light mounted at the spin stand. Each user could also adjust the

position and focus of the camera (PCO Pixelfly) as required. Once the optical system was operating as required, the disc is stopped and liquid was then loaded for testing.

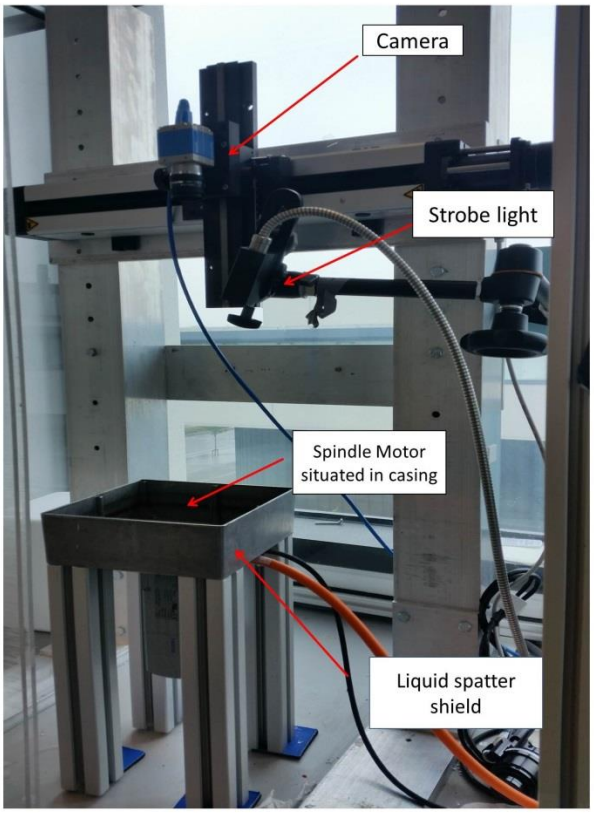

Figure 16: Test spin stand set-up.

3.3 Detection of DNA by LAMP

3.3.1 Removal of Potential DNA Contaminants

All sample preparation was carried out in UV hood (UV4PCR, Fisherbrand, Ireland). This UV hood provides efficient decontamination of equipment before carrying out any sensitive DNA amplification reactions, minimising the risks of contamination of DNA samples. The PCR hood is first decontaminated with 30 minutes of UV radiation prior to sample preparation.

3.3.2 Preparation of Materials for LAMP Reaction

All DNA samples used throughout this work were obtained from IDT (Integrated DNA Technologies, Belgium) as advised by our collaborators in Wageningen University and Research, Wageningen, The Netherlands; and National Institute of Biology, Ljubljana, Slovenia. The two main DNA samples used throughout this work were sequences from the Tomato Leaf Curl Virus (TYLCV) and the fungus Botrytis Cineria (BOTY) in the form of G-Blocks.

G-Block samples were received in a dehydrated form and were rehydrated as per the manufacturer's instructions to a final concentration of 10 ng / µL. G-Block samples were vortexed and centrifuged briefly to ensure the dehydrated pellet was at the bottom of the vial before rehydration. Each dehydrated G-Block sample was rehydrated with 50 µL 1x Tris-EDTA Buffer (Tris-EDTA Buffer 100x Concentrate, Sigma-Aldrich, Ireland). The 1x concentration stock was made in house using Nuclease-free water (Water Nuclease Free, VWR, Ireland). The G-Block sample was then placed in a temperature controlled oven at a temperature of 50 ˚C for 20 minutes. Finally the G-Block sample was briefly vortexed and

centrifuged for a final time. This was then divided into 10 µL aliquots and stored in a -20 ˚C freezer until time of use.

The Target Primers were ordered and received from Integrated DNA Technologies (Integrated DNA Technologies, Belgium). The Target Primers were received dehydrated and rehydration was performed as per the specification sheets included in the delivery. Each Target Primer was rehydrated with a specific volume of nuclease-free water that yielded a final concentration of 100 mM. The Target Primers were then left to stand on bench for 30 minutes to ensure efficient rehydration had occurred. They were then divided into 100 µL aliquots and stored in a -20 ˚C freezer until time of use. As advised by our partners a concentration of 40 µM was recommended for use in each sample reaction. The addition of nuclease-free water was used to obtain the desired concentration.

The Isothermal master mix ISO-001 was received from Optigene (Optigene, UK). No other preparation was required upon delivery and was stored in a -20 ˚C freezer until time of use.

All biological samples were stored in a -20 ˚C freezer when not in use. During reagent/sample preparation all components were kept on ice. Samples were separately rehydrated and aliquoted in an irradiated PCR hood to prevent cross contamination.

The sample reaction was prepared as outlined in Table 2.

Sample Preparation		
	1x (µL)	25x (µL)
Isothermal Mastermix	15	375
FIP (40 µM)	1.25	31.25
BIP (40 µM)	1.25	31.25
B3 (40 µM)	0.125	3.125
F3 (40 µM)	0.125	3.125
LF (40 µM)	0.75	18.75
BF (40 µM)	0.75	18.75
G-Block Template	1	25
Nuclease-free water	4.75	118.75
Total	25	625

Table 2: Preparation of samples for a 1x and 25x reaction. The reagents highlighted in yellow are the target primers specific to each G—block template.

Where non-template controls (NTCs) are used throughout the course of this work the same volume of nuclease-free water is used in place of the G-Block template.

3.3.3 DNA Amplification and Detection

DNA amplification was detected out using a number of different methods throughout this work. The various methods, materials and instruments used in this process are described in the following sections.

3.3.3.1 Rotorgene

Verification of amplification conditions were initially confirmed off-disc using a Corbett Rotorgene 6000. Samples were prepared in a UV irradiated hood (see Section 3.3.1) using DNAase free PCR tubes and DNAse free filtered pipette tips. All steps were carried out on ice. A master mix was first prepared according to table 2 and aliquoted into the PCR tubes. Samples were then added to the appropriate tubes. Non-template controls were prepared using the nuclease free water used to prepare the master mix.

The amplification method used throughout this work, Loop-mediated isothermal amplification (LAMP), which uses isothermal conditions of exposing the DNA sample to temperatures between 60 ˚C – 65 ˚C for the duration of 1 hour [100][61]. A Thermal cycler was used to create these conditions required for LAMP to take place. For this work, the conditions were set at 65 ˚C, recording fluorescence on the SYBR green channel every 30 seconds for up to 1 hour.

After LAMP was completed a melting curve was generated using the Rotorgene 6000 Series software as per the manufacturer's instructions.

3.3.3.2 Gel Electrophoresis

In later stages of the project the amplification of samples took place on disc. The microfluidic disc containing samples was sealed with clear PSA to avoid any loss of samples through evaporation and wrapped in tinfoil before being placed into a laboratory oven. The temperature of the oven was set at 70 ˚C, to account for the poor thermal transfer of the PMMA and the laboratory oven, and the disc was heated for a total of 1 hour. The disc was then removed.

Samples were subsequently stored at 4 °C until analysed by agarose gel electrophoresis. For this work, verification of LAMP products was carried out on a 1 % agarose gel (Agarose, Sigma-Aldrich, Ireland). The 1 % agarose gel was made by adding 1 g of agarose to 100 mL of 1x TAE buffer (Tris Acetate-EDTA Buffer, Sigma-Aldrich, Ireland) placed into a microwavable flask and heated for 1-3 minutes. The agarose must be completely dissolved in the 1x TAE buffer. The agarose solution was left to stand and cool for a minimum of 5 minutes. This was then poured into a gel mould (Horizontal Gel Apparatus, BIORAD, UK) and stored in a fridge until ready to use. Each agarose gel should be made fresh and can only be kept in the fridge for a maximum of one day.

5 µL of loading dye was mixed with 1 µL of sample before loading onto the gel. This contained loading dyes- bromophenol blue (0.25 %) or xylene cyanol (0.25 %) and Ficoll (type 400) (15 %) (Sigma-Aldrich, Ireland). On a 1 % agarose gel bromophenol blue and xylene cyanol migrate approximately with 300 bp and 4000 bp fragments respectively. Ficoll is added to increase the density of the sample to allow the DNA sample to sink to the bottom of the well. The loading dyes are used to monitor the progress of the sample during electrophoresis. A Hyperladder™ 1 KB (Bioline, UK) was also loaded onto the gel in parallel with the samples. This is a molecular weight marker especially designed for DNA size determination.

A power supply (Powerstation 300, LABNET, USA) was used to apply the electric current required for gel electrophoresis to take place. A current was applied to the agarose gel at 100 Volts for 30 minutes to ensure the sample did not run off the end of the gel thus resulting in an unreadable gel.

The agarose gel was stained for a minimum 20 minutes in 10 µg/mL ethidium bromide (Ethidium Bromide, Sigma-Aldrich, Ireland). This stain is commonly used for detecting DNA

in gel electrophoresis which inserts into the grooves of the DNA and this fluoresces under UV light. Extreme caution must be used during the staining process as ethidium bromide is considered carcinogenic. Gloves must be worn at all times during the staining process.

A UV Imager (MiniBIS Pro, DNR bio-imaging systems, Israel) was then used to analyse the stained gel. UV imagers provide real-time image viewing and analysis with a scientific high-resolution camera. The gel was placed on the sliding drawer and inserted into the machine. The gel was analysed and images were acquired using GelCapture and DNR's acquisition and analysis software. The exposure and gain were adjusted to obtain the clearest image using this software.

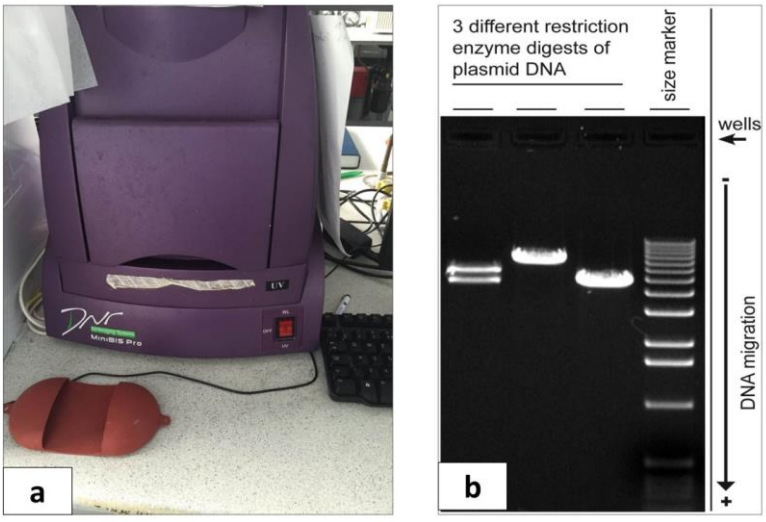

Figure 17: UV imager and DNA bands from gel electrophoresis. a) The UV imager used throughout this work to analyse gels. b) A typical image resulting from gel electrophoresis [101].

Chapter 4

2x3 Combinatorial Mixing

Combinatorial Mixing of Samples with Target-Specific Primers for Rapid Pathogen Detection on a Centrifugal Platform

4.1 General Introduction

As previously discussed in Chapter One screening methods for the detection of foodborne pathogens are developing and are of great interest to the scientific community. One method, Loop-mediated isothermal amplification (LAMP), has shown great promise over the last few years as a reliable method of DNA amplification due to its high specificity and efficiency under isothermal conditions [61]. LAMP is the amplification method of choice used throughout the course of this work.

The integration of DNA amplification methods on centrifugal microfluidic devices has gained significant interest for deployment for on-site applications. However, there still exists a need for centrifugal microfluidic devices to test for a number of different sample types against a variety of different active agents. Across a wide range of biological and chemical sciences, the capability to screen a large number of samples (M) for specific responses to a library of active agents (N) is of critical importance. However, a major challenge is that, as the number of samples or number of active agents in the library increases, the number of benchtop liquid handling steps required increases by the scalar

dimension. For example, increasing the number of 'M' samples by one (to 'M+1') increases the number of liquid handling steps from 2xMxN to 2x(M+1)xN thus requiring 2xN more liquid handling steps. If N should be large this can have a major increase on the throughput of a conventional laboratory platform.

We have developed a novel centrifugal microfluidic (lab-on-a-disc (LOAD)) approach for performing screening studies while minimising the number of liquid handling operations required. A centrifugal microfluidic disc with the ability to mix two samples against three active agents, in this case three different sets of LAMP primers, is demonstrated. This disc is enabled using centrifugo-pneumatic dissolvable film burst valves. On disc mixing and LAMP amplification is successfully demonstrated.

4.2 Disc Manufacture and Assembly

The microfluidic disc (Ø 120 mm) was manufactured and assembled as described by the standardised methods in Section 3.1.2-3.1.4. The microfluidic disc was assembled using four layers of PSA and four layers of PMMA. Laser ablation methods were used to create chambers and through-holes in the PMMA layers while a precision knife-cutter was used to create small connecting features in the PSA layers.

The purpose and application of each layer is described in detail in Section 3.1.4. Two PMMA thicknesses were used in the manufacture and assembly of this disc – 1.5 mm PMMA and 0.3 mm PMMA. The laser machine cutting settings for each PMMA thickness is given in Table 1. Layer 3, which contained the reservoirs, was manufactured with 1.5 mm PMMA to provide the depth to load and store reagents. Layers 1, 6 and 8 were manufactured with 0.3 mm PMMA. The 0.3 mm PMMA provided very thin layers to allow heat penetration during

isothermal testing of the samples on disc. The PMMA thickness for each layer is outlined in Table 3.

PMMA thickness is disc manufacture		
PMMA (mm)	0.3	1.5
Layer no.	1, 6, 8	3

Table 3: The various PMMA thickness used in the manufacture of the 2x3 microfluidic disc.

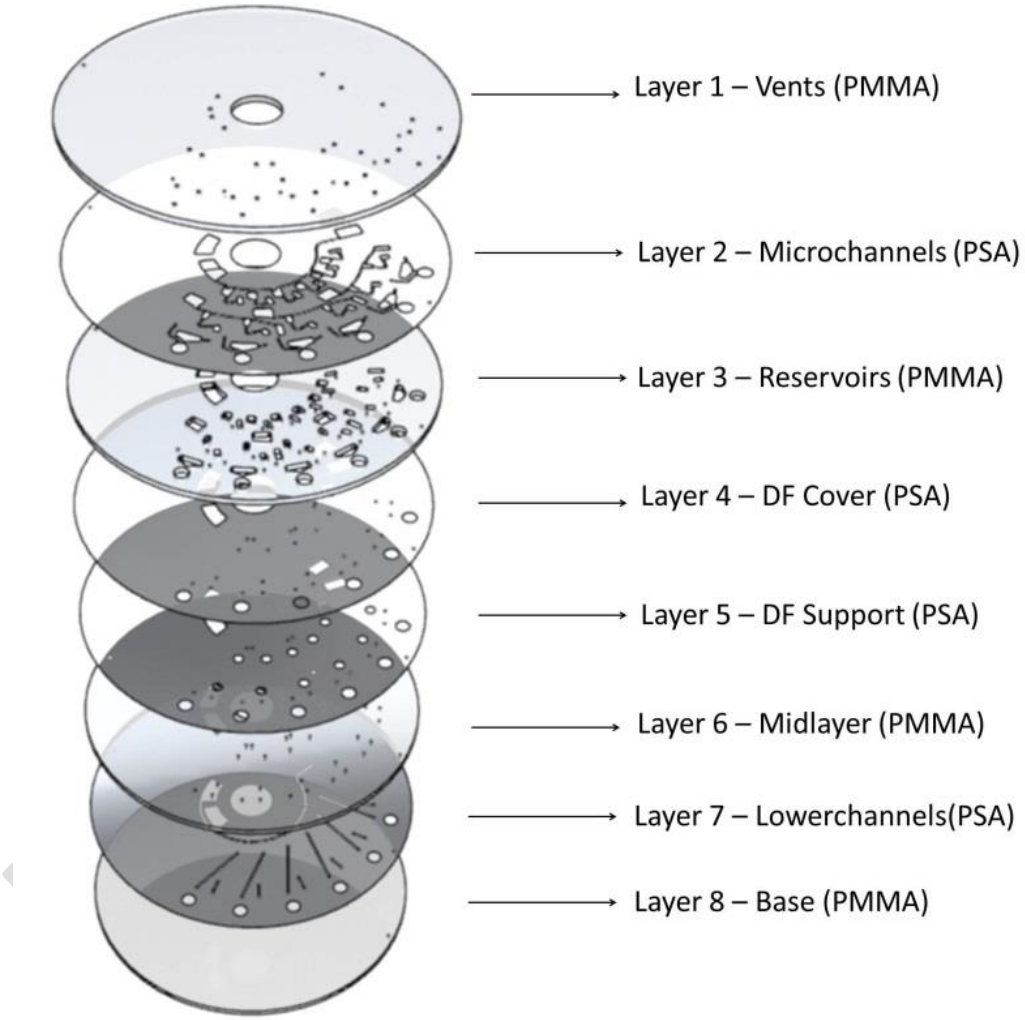

Figure 18: Detail of disc assembly. A total of eight layers included in the disc assembly which allowed a multi-layer architecture. DF tabs were carefully placed between layers 4 and 5. Each PSA and PMMA layer were rolled a minimum of five times and rotated between passes.

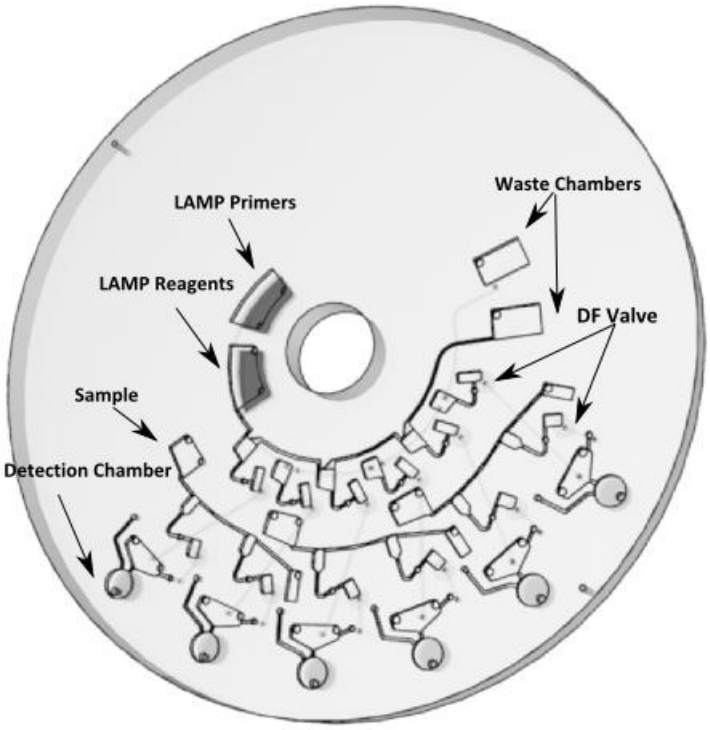

Figure 19: The complete assembly of the 2x3 microfluidic disc with the labelled key structures. The LAMP primers and LAMP reagents are loaded into their respective chambers and metered into three separate aliquots each. The three sample chambers are loaded and metered into two separate aliquots each. Waste chambers are located at the end of every loading chamber to capture any excess liquid. All reagent components are collected in the detection chamber once all DF tabs have burst.

4.3 Working Principle of Disc

The microfluidic system was tested fluidically with the use of coloured food dyes as a visual representation of the various samples and reagents used on the system. The disc was mounted onto the spindle motor on the spin stand and securely fit with a screw. The PCO and LabVIEW software were initialised as described in Section 3.2. Fluidic analysis was carried out by applying different spin frequencies to the microfluidic disc to determine the optimum spin frequency allowing efficient metering of all chambers without causing the DF valves to open.

Spin Frequency Protocol		
Liquid Handling Step	Hz	Time (s)
Metering	20	40-45
Liquid in contact with DF tab	60	3-4
Liquid collected in Detection Chamber	60	<5

Table 4: The final spin frequency for the full operation of the 2x3 microfluidic disc.

The final spin frequency protocol was determined. The initial spin frequency was set at 20 Hz to allow efficient metering of all chambers without the DF valves prematurely opening. This spin frequency was applied until all chambers had metered completely. The spin frequency was then increased to 60 Hz to allow the food dye to overcome the pressure in the pneumatic chamber. The DF was wetted, dissolved and the food dye was collected in the detection chambers.

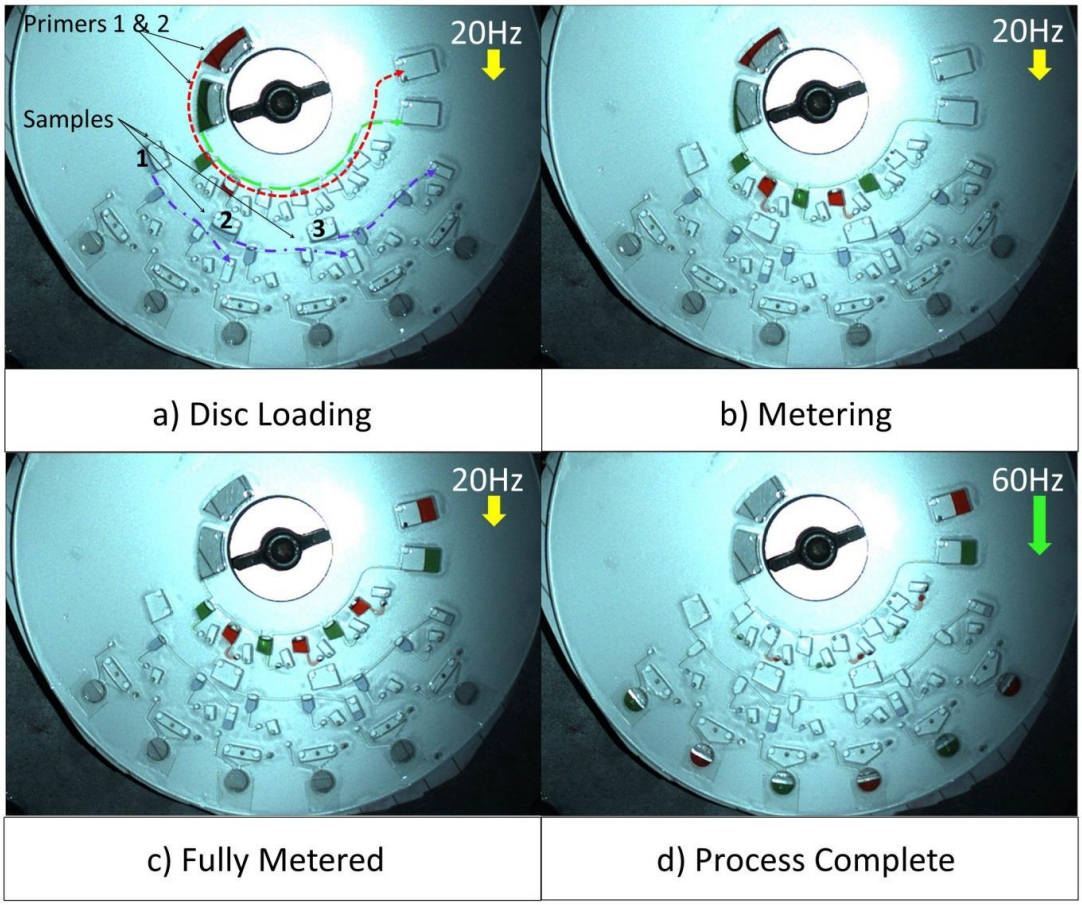

Figure 20: Fluidic testing of 2x3 microfluidic disc. The various coloured food dyes are pre-loaded onto the disc and spun at a frequency of 20 Hz. Coloured food dye is used to provide a visual representation of different reagents and samples tested throughout this work; red- BOTY primer 1, green- TYLCV primer 2, Blue- NTC sample 1, BOTY sample 2 and TYLCV sample 3. (a) Each sample (samples 1 to M) loaded on the disc is divided into 'N' aliquots while each of the primers (primers 1 to N) loaded onto the disc is divided into 'M' aliquots. (b-c) Once metering is completed, the disc spin rate is increased to open the DF burst valves. (d)The liquid elements are then pumped, through a multi-layer architecture, to reservoirs on the periphery of the disc where each individual sample mixes with an individual reagent to create MXN unique reagent combinations.

4.4 Dissolvable-Film Valves

The theory of DF tabs is described in Chapter 2 and the manufacturing process is outlined in Section 3.1.2.3.

A total of twelve DF tabs were positioned on the microfluidic disc as seen in Figure 21. Embroidery film was used in the manufacture of the DF tabs and dissolves in 3 seconds upon contact with water. Six DF tabs were placed closer to the centre of the disc and another six positioned nearer the edge of the disc

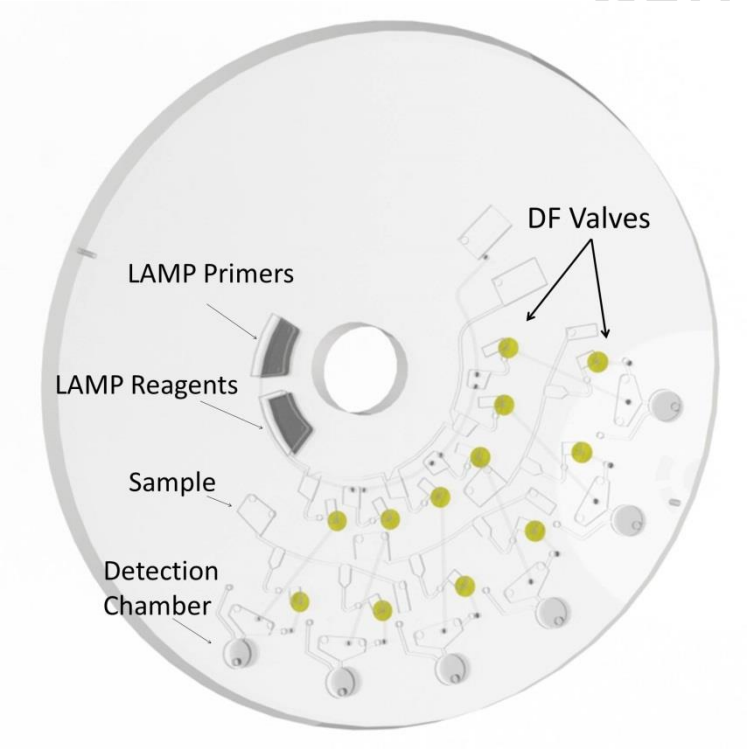

Figure 21: The positions of the DF tabs on 2x3 microfluidic disc are highlighted in yellow. There are six DF tabs situated close to the centre of the disc and six DF tabs situated at a location nearer the edge of the disc.

4.4.1 Working Principle of DF Valves

The theory of how DF valving systems operate was outlined in the literature in Section 2.2.1. The integration of DF valves into this microfluidic disc enabled precise flow control and reagent isolation. Centrifugo-pneumatic valving system was used throughout the operation of this microfluidic disc. The DF tabs form a liquid barrier which seals a through hole connecting two fluidic layers. The DF tabs were manufactured as described in detail in Section 3.1.2.3.

The burst frequency of the valves can be calculated using the equations previously explained in Section 2.2.1. As shown in Figure 21, there are twelve DF tabs positioned in the disc with six positioned closer to the centre of the disc and six positioned towards the outer periphery of the disc. The burst frequency can be determined for each valve using the equation

$$\omega = \sqrt{\frac{P_0\left(\frac{1}{1-\frac{\Delta V}{V}}-1\right)}{\rho \Delta r \bar{r}}} \qquad (4.1)$$

The density is represented by ρ, Δr is the radial length, $\bar{r}$ is the mean position of the liquid plug ,ω is the angular frequency, ΔV is the volume of the liquid plug and V is the volume of the pneumatic chamber.

The values used in the equation are contained in the table 5.

Values for calculating burst frequencies		
	Inner DFs	Outer DFs
ρ (kg m-^{3})	1000	1000
ΔV ($^{m-3}$)	1.5E-09	1.5E-09
V (m^{-3})	1.02E-08	1.05E-08
r_0 (m)	0.02001	0.033
r_1 (m)	0.02572	0.03918
Δr (m)	0.00571	0.00618
$\bar{r}$ (m)	0.022865	0.03609
P_0(Pa)	101326	101326
ω $(Rad\ s^{-1})$	365.7992	275.1661
Hz s^{-1}	58	43

Table 5: Design parameters and predicted burst frequencies

The appropriate values, given in Table 5, were used in the equation above to calculate the burst frequencies for both the inner and outer DFs. The ω value was given in radians and so was converted to Hz by

$$\frac{\omega}{2\pi} \qquad (4.2)$$

From applying these equations the burst frequency for the inner and outer DFs were determined. The burst frequency for the inner DFs was 43 Hz and the inner DFs was 58 Hz.

The volumes and geometries were so designed to allow the metering of all sample/reagents at 30 Hz. This was efficiently carried out to allow accurate metering of sample/reagents and without any premature bursting of DFs. The spin frequency was then increased to 60 Hz to

allow the DFs to burst and the transport of sample/reagents was then complete. No difficulty was observed when increasing the spin frequency to 60 Hz and this spin frequency allowed successful bursting of the DFs both located towards the centre and the periphery of the disc.

From extensive research of the literature and from the high level of expertise from the Microfluidics Research group I determined that DF Burst valves were the best type of valving system to integrate into this disc design. The ΔV volumes were selected by determining the smallest size the laser cutter could cut with great precision and reliability. Next the location and height of the fluid elements, $\Delta r \; and \; \bar{r}$, were determined. This was determined based on the knowledge that the burst frequency was required to be in the range of 30-60 Hz. Based on these inputs I selected a number of volumes, V, for the DF Burst valves and calculated the burst frequencies. The end-point volumes selected were within the 30-60 Hz range and were also based on the available space on the disc. A wide range of burst frequencies could be utilised for this particular disc design as it is a single stage disc. However, the burst frequencies would have to be tailored with greater accuracy if this disc was designed for the purpose of a multi-stage disc such as an ELISA.

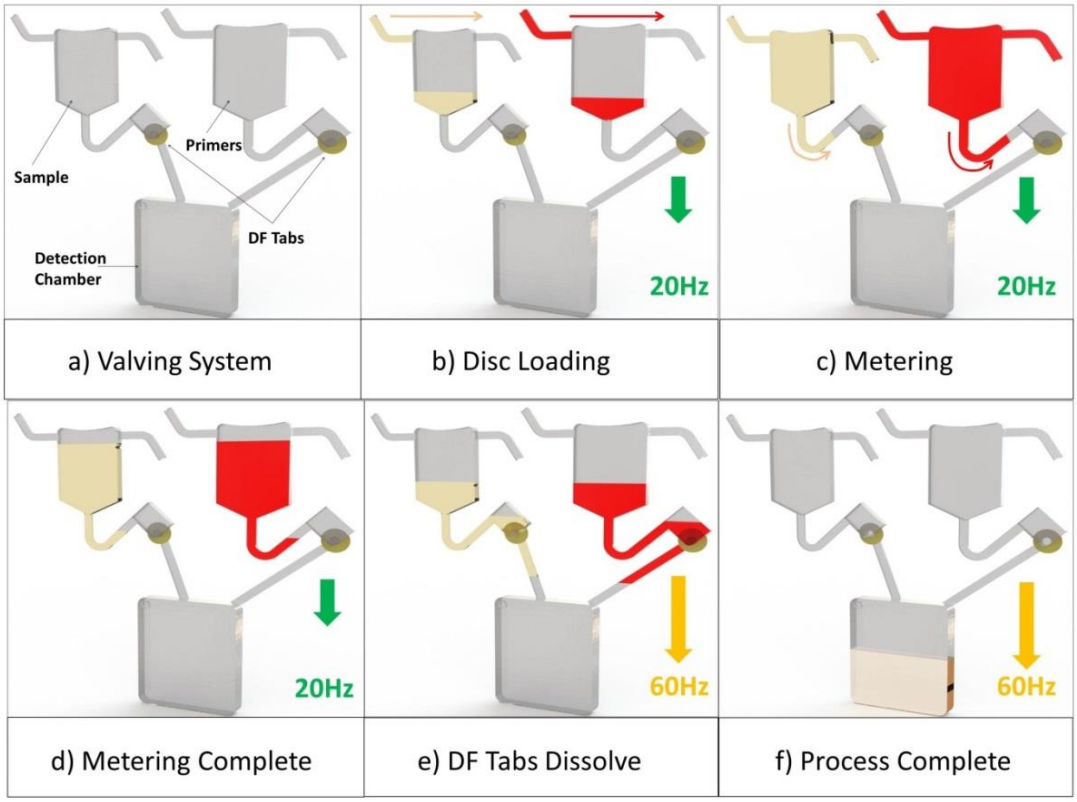

Figure 22: A schematic of the centrifugo-pneumatic valves. a) A sample, primer and detection chamber is shown. The positions of the DF tabs are highlighted. The sample (yellow) and primers (red) are loaded onto the disc at a spin frequency of 20 Hz. b) The sample and primer enters their relevant chambers through a metering channel. c) The sample and primers entirely fill the chambers and exit through a metering channel to progress to the next chamber. Air is trapped in the pneumatic chamber which stops the sample/primer coming into contact with the DF d) Metering of the sample and primers are complete. e) Increased spin frequency to 60 Hz induce air compression and air/fluid inversion on the chamber. The sample and primer come into contact with the DF causing it to dissolve and the vale opens. f) The sample and primers are collected in the detection chamber and are mixed.

4.5 Biological and Material Optimisation

During the course of this work various optimisation steps were carried out to achieve reliable DNA amplification on disc. This was carried out with regards to biological and material aspects.

4.5.1 Materials Compatibility

4.5.1.1 On-Chip Testing

A simple chip was used in the initial testing of DNA amplification. An eight chamber chip was manufactured to simulate the chambers located on the edge of the disc. This was carried out to identify any materials compatibility issues at an early stage. Three different DNA samples, which are pathogenic to tomatoes, were loaded onto the chip which included:

- Botrytis Cinerea (BOTY), a fungus
- Tomato Yellow Leaf Curl Virus (TYLCV), a single-stranded DNA virus
- Clavibacter Michiganensis (CMM), a bacterial pathogen

Two BOTY, TYLCV and CMM samples were loaded onto the chip with the addition of two non-template controls (NTCs). The DNA samples were prepared as described in Section 3.3. The addition of mineral oil was included to each sample to decrease the likelihood of any loss of sample due to evaporation. Clear PSA was used to seal the loading holes of the chambers to avoid cross contamination of samples and as another precautionary measure to decrease the loss of sample due to evaporation.

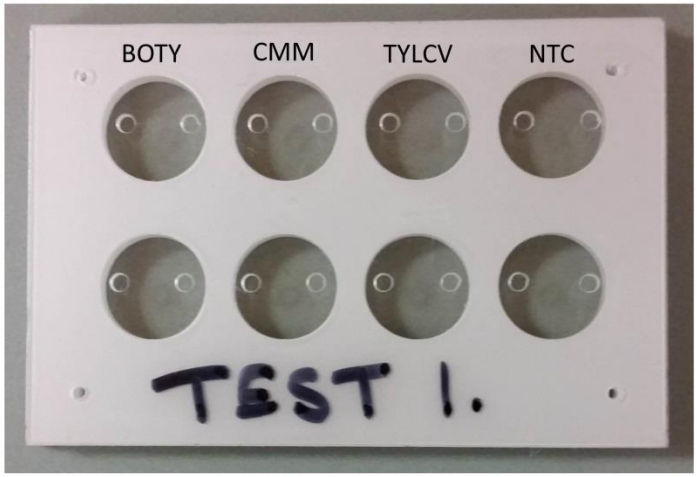

Figure 23: An assembled test chip used for initial amplification testing. This included eight reaction chambers replicating those on the microfluidic disc. Two BOTY, CMM and TYLCV samples were loaded in addition to two NTCs.

In parallel, three of each of the DNA samples was loaded into the microfuge with additional non-template controls (NTCs). The chip and the microfuge tubes both containing the DNA samples were placed in a standard laboratory oven at 70 ˚C which replicated LAMP conditions for a total time of1 hour. Each microfuge tube was wrapped in a small piece of foil before being placed in the oven. After 1 hour the samples were collected and loaded onto a gel for DNA amplification confirmation by gel electrophoresis. The samples were run on a 1 % agarose gel and an electrical current was applied at 100 volts for 30 minutes as described in Section 3.3.3.2. The gel was then observed with a UV imager (as described in Section 3.3.3.2). Positive results were obtained from the samples placed into the microfuge tubes while the samples loaded onto the chip showed no DNA amplification. This indicated a possible inhibition of DNA amplification caused by the surface of the PMMA due to possible surface particulates. As a next step, various surface treatments were applied to the PMMA to eliminate any surface particulates and possibilities of the surface of the PMMA inhibiting DNA amplification.

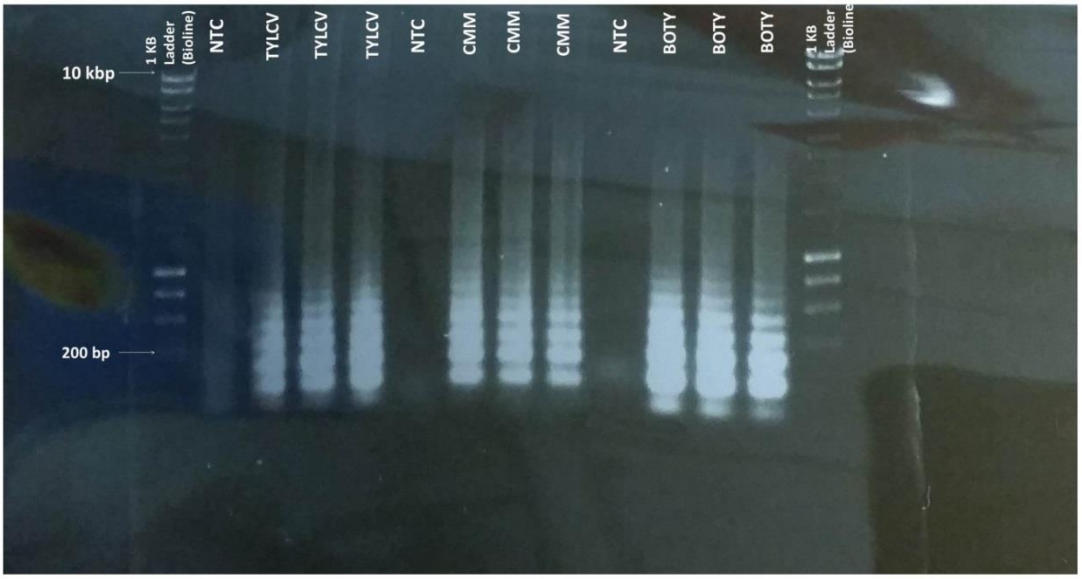

Figure 24: Amplification of DNA samples contained in PCR tubes in oven. DNA samples and non-template controls (NTCs) loaded into the PCR tubes with small pieces of PMMA and placed in an oven at 70 ˚C for 1 hour. Confirmation of DNA amplification by gel electrophoresis is represented by long smeared bands. BOTY, CMM and TYLCV refer to names of the DNA used in this test. 1 KB DNA ladder (Bioline) was used as a comparison of the size of the DNA band widths. NTCs did not amplify showing reliable DNA amplification of DNA samples.

4.5.1.2 Surface Treatment of PMMA

The surface treatment of PMMA was carried out to eliminate any residue or particulates, possibly caused by laser ablation process that may inhibit the DNA amplification. The following surface treatments were carried out:

1. Untreated PMMA
2. MICRO90 Washed PMMA (Given in Section 3.1.3.1)
3. MICRO90 Washed and BSA Blocked PMMA (Given in Section 3.1.3.2)

The addition of BSA to the washing protocol was due to its widespread use for relieving interference in PCR and other DNA amplification methods and is a cost-effective additive

for this purpose [102]. BSA created a coating on the PMMA surface which can often inhibit the polymer from absorbing the sample. The concentrations of BSA used throughout the washing protocol were advised by a colleague with prior experience. Using this concentration provided a sufficient improvement in performance to meet the projects goals. Therefore, it was determined that further investigation was outside of scope.

Small pieces of PMMA were treated according to the conditions outlined above. These small pieces of PMMA were added to a small microfuge containing the DNA or non-template control (NTC) sample. The DNA sample used during the surface treatment test was BOTY. Sixteen samples were tested on the thermal cycler with the addition of different treated pieces of PMMA. The samples were prepared as follows:

Sample set up for treated PMMA	
Sample Type	**Number of samples**
NTC Only	2
NTC + Untreated PMMA	2
BOTY Only	3
BOTY + Untreated PMMA	3
BOTY + MICRO90 Treated PMMA	3
BOTY + MICRO90 & BSA Blocked	3

Table 6: Sample set up for various surface treatments of PMMA

The DNA samples were prepared as described in Section 3.3.2.

The NTCs and DNA samples with/without the addition of small pieces of PMMA were placed into the thermal cycler at 65 ˚C for a total time of1 hour. The results obtained are shown below.

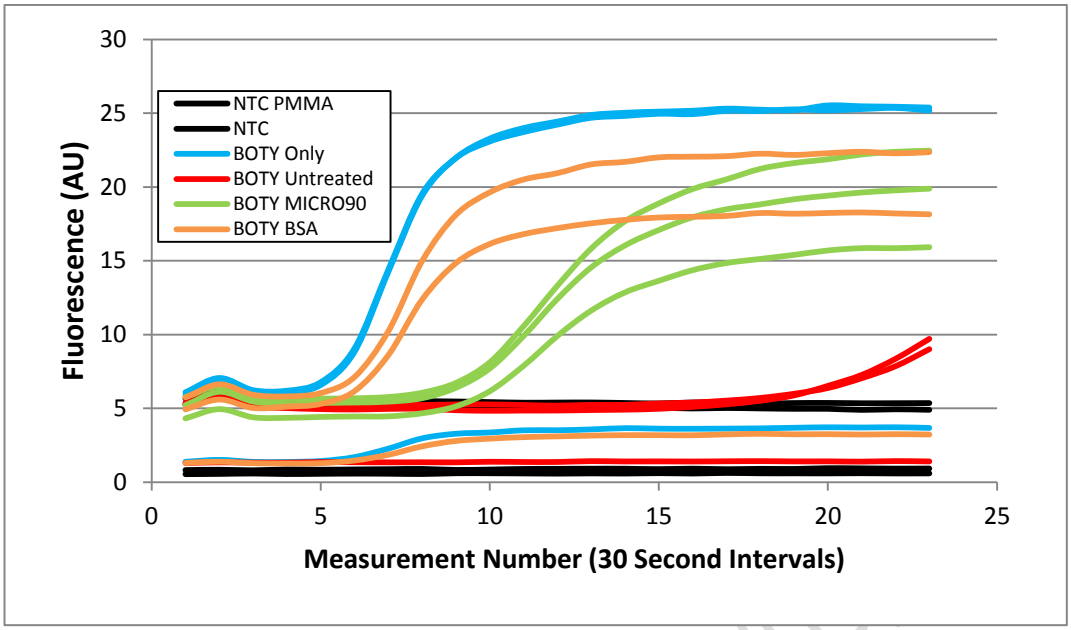

Figure 25: Materials Compatibility. Addition of treated PMMA to DNA samples. The results obtained from the addition of treated PMMA to the DNA samples. Sample measurements were recorded at 30 second intervals. The untreated PMMA (red) showed no amplification indicating pre-treatment of PMMA was required to permit amplification to occur. The MICRO90 washed PMMA (green) and MICRO90 with addition of BSA blocked PMMA (orange) showed amplification occurred. The samples were prepared as described in Section 3.3.2. Amplification and measurements were carried out on a Rotorgene 6000 as per Section 3.3.3.1.

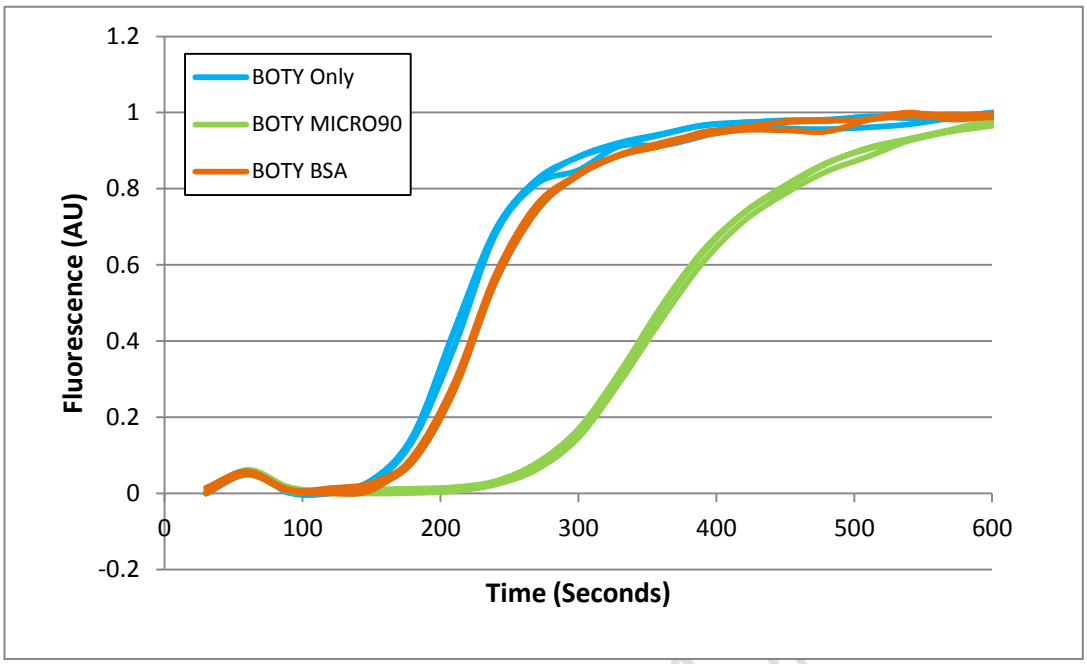

Figure 26: Materials compatibility normalised. This shows the amplification of samples when different surface treatments were applied. The PCR tubes with no addition of PMMA (blue) amplified first. The PCR tubes with the addition of PMMA treated with MICRO90 and BSA blocked amplified next. The PCR tubes with the addition of PMMA treated with MICRO90 only amplified last.

From the results obtained using the thermal cycler it could be concluded that the addition of untreated PMMA inhibited the amplification of DNA (shown in red) in Figure 25 and more clearly in the normalised graph in Figure 26. With the addition of MICRO90 treated PMMA DNA amplification occurred (shown in green). However, the best result was obtained with the addition of MICRO90 + BSA blocked PMMA (shown in orange) as the amplification times occurred at a faster rate than the PMMA treated with MICRO90 only.

The results are summarised in Table 7.

Materials Compatibility Results			
	Sample Number		
	1	2	3
NTC	x	x	x
NTC+Untreated PMMA	x	x	x
BOTY Only	✓	✓	✓*
BOTY+Untreated PMMA	x	x	x
BOTY+MICRO90 Treated PMMA	✓	✓	✓
BOTY+MICRO90&BSA Blocked PMMA	✓	✓	✓*

*Table 7: A summary of the results obtained from the treated PMMA test. *Amplification occurred but the relative fluorescence is low. This could have been a result from the presence of air bubbles or a low volume sample (pipetting error).*

4.5.1.3 Test Chips

Test chips were manufactured to initially test the surface treatment of PMMA during the assembly process. The surface treatments were applied to the chips shown in Section 4.3.1.2. The two surface treatments applied to the chips were the MICRO90 washed PMMA (described in Section 3.1.3.1.) and MICRO90 washed and BSA blocked PMMA (described in Section 3.1.3.2.). Assembly was carried out as described in Section 3.1.4.

A total of six chips were tested: Three chips treated with the MICRO90 wash only and three chips treated with the MICRO90 and BSA blocked wash. A number of conditions were carried out during this test:

Test Conditions on Chips	
MICRO90 only treated PMMA	Micro90 & BSA Blocked treated PMMA
Heat applied + food dye	Heat applied + food dye
No heat applied + food dye	No heat applied + food dye
Heat applied + no food dye	Heat applied + no food dye

Table 8: Test Chip conditions. Six chips were tested using two different surface treatments as described in Sections 3.1.3.1 and 3.1.3.2. The assembly process was carried out as described in Section 3.1.4.

The chips with heat applied were placed in a standard laboratory oven at 70 ˚C for a total time of 1 hour. Only one chamber was filled to the half-way point with red food dye. The other chambers were filled with DI water. This was carried out to observe if any red food dye travelled to other water filled chambers. The chips which did not experience any heat were placed on the bench.

After the duration of 1 hour the chips were observed and compared. The two chips surface treated with MICRO90 and BSA blocked showed significant leaking of food dye from their

respective chambers. The chips could also be readily separated by hand, demonstrating further the inefficient bonding of the PSA to the PMMA surfaces. The chips surface treated with MICRO90 only showed no signs of leaking. This suggested that the BSA coating on the PMMA layers provided ineffective sealing, and therefore leaking occurred. The addition of BSA for optimal DNA amplification was therefore integrated using a different approach (Section 4.3.1.4.).

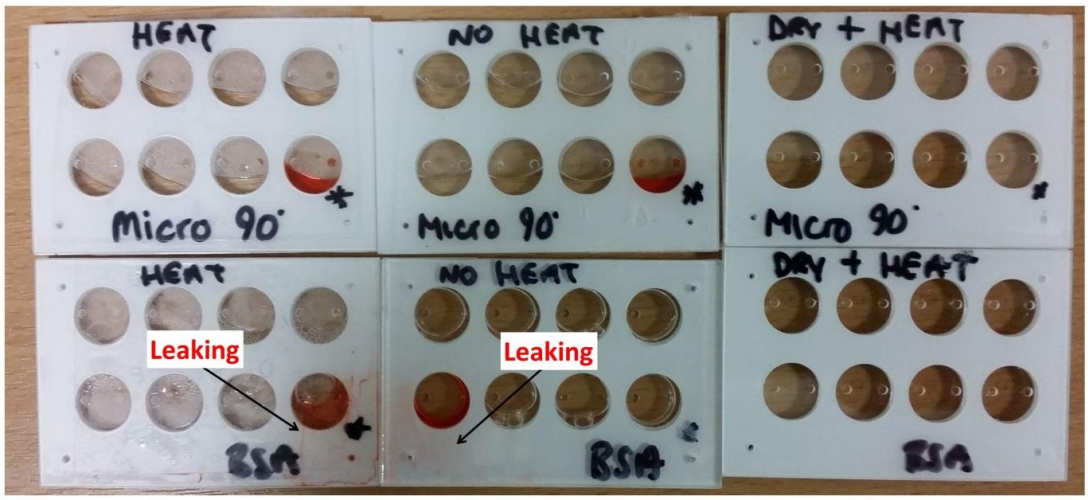

Figure 27: The six test chips used in the surface treatment test. The bottom left and centre chips treated with MICRO90 and BSA blocked showed significant leaking from the chamber filled with red food dye. The chips top left and centre showed no signs of leaking when PMMA was surface treated with MICRO90, only.

4.5.1.4 Addition of BSA Solution to DNA Sample

As significant leaking was observed when the PMMA was surface treated with the MICRO90 and 3 % BSA solution, it was determined whether adding the BSA solution directly into the DNA sample would interfere with amplification. For previous DNA amplification testing the sample reaction was prepared as described in table 2 (Section 3.3.2.) For these set of tests,

the 3 % BSA solution was added into the sample reaction in place of the nuclease free water.

This test was carried out using the Rotorgene 6000 (Corbett). BOTY was used as the DNA sample during this test. The samples were prepared as shown in Table 9.

Preparation of samples for BSA testing		
	Reaction (nuclease-free water)	Reaction (3 % BSA)
BOTY	3	3
NTC	1	1

Table 9: Preparation of samples for BSA testing. Samples were prepared as described in Section 3.3.2. Samples with the addition of 3 % BSA solution in place of nuclease-free water were prepared in parallel. An NTC was prepared for each sample condition- one with nuclease-free water and one with 3 % BSA solution.

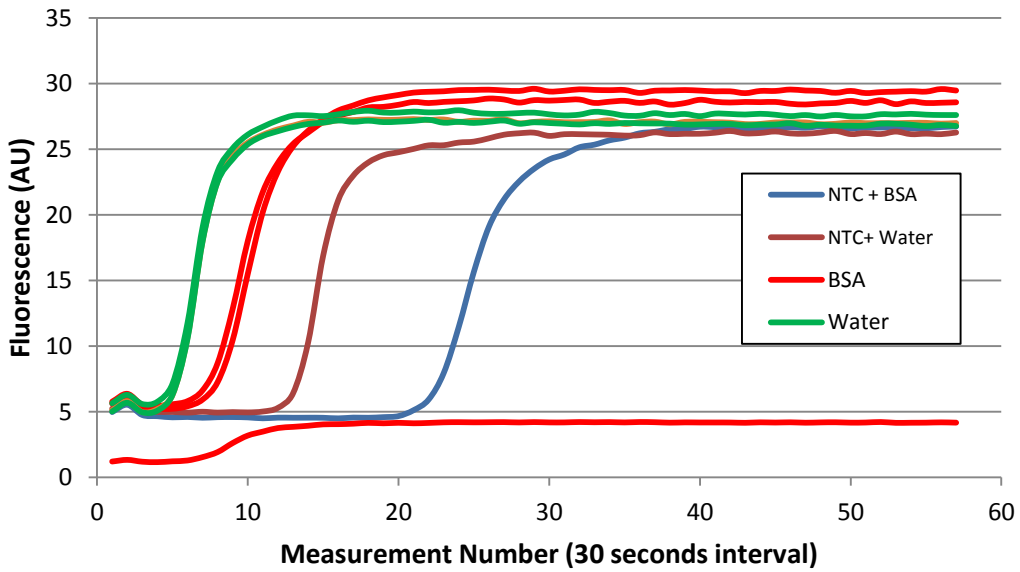

Figure 28: Addition of BSA into sample. Results obtained from thermal cycler for BSA test. The water samples (green) show fastest amplification and the BSA samples (red) also show positive amplification. NTCs show positive amplification after some delay, implying that much of the *amplification seen in the sample is due to the sample itself being positive rather than the contamination of reagents.*

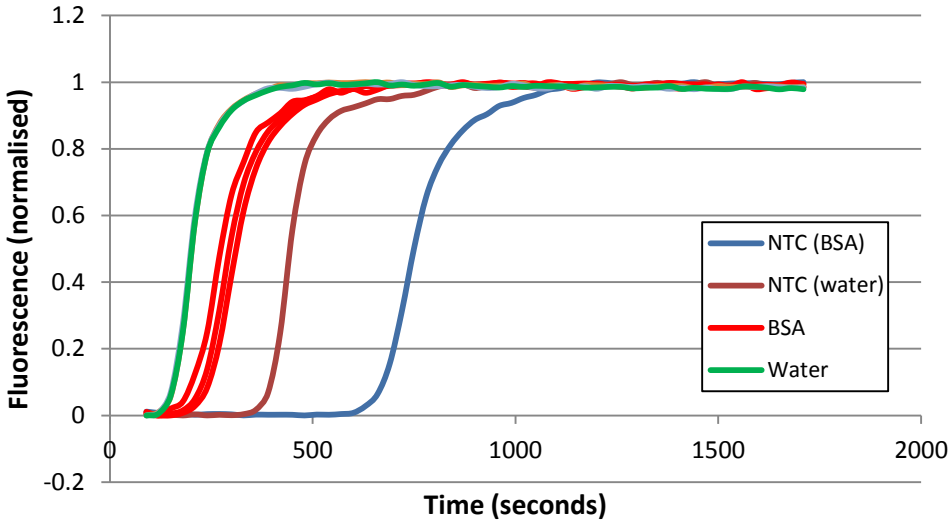

Figure 29: Addition of BSA into sample normalised. Normalised results obtained from BSA test. The addition of BSA directly into the sample allows amplification to occur. The samples with the addition of water and the samples with the addition of BSA occur at similar times indicating that no inhibition of sample amplification has occurred.

It could be concluded that the addition of 3 % BSA did not inhibit the DNA amplification and could be integrated into the sample preparation in place of the nuclease-free water. This will reduce any loss of sample from absorption to the PMMA surface.

4.6 Operation of Disc

4.6.1 Working Principle of Disc

This microfluidic disc was designed to permit combinatorial mixing of samples (M) with active agents (N). This is highly advantageous compared with testing of one single sample against a number of different active agents. The following simplified schematic shows the working principle of combinatorial mixing. LAMP was demonstrated with two DNA samples + one NTC (M=3) for the presence of two pathogens (N=2). BOTY and TYLCV DNA samples were used throughout this test. DNA amplification only occurs when a DNA sample is mixed

with its specific target primers i.e. BOTY sample mixed with BOTY primers. Mixing of a sample with other primers does not permit DNA amplification to occur. In the schematic below the red indicates where no DNA amplification occurred and the green indicates the occurrence of DNA amplification.

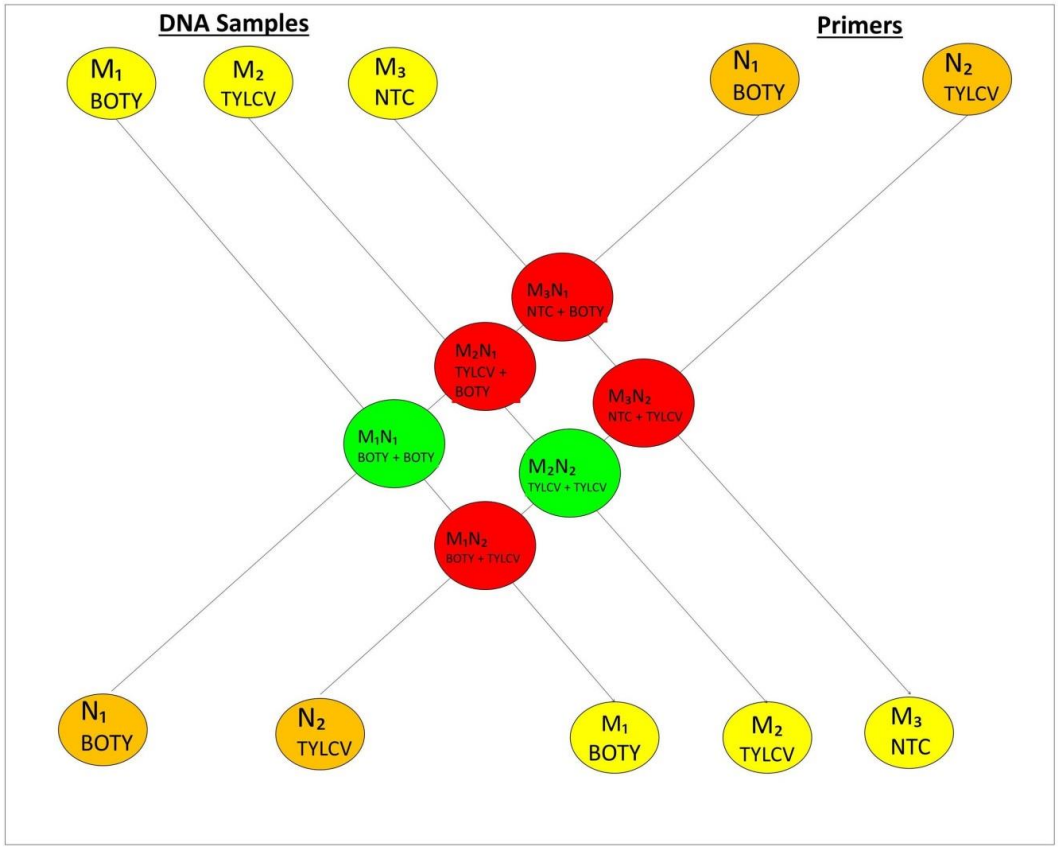

Figure 30: Schematic showing the combinatorial mixing principle. Samples (M) are shown in yellow and primers (N) shown in orange. Cross-mixing of sample/primer occurs at the intersection of the channel path. Expected DNA amplification is indicated by the colour green and no DNA amplification is indicated by the colour red.

Combinatorial mixing allows a significant reduction in pipetting steps often encountered by analysis of a large number of samples against a wide range of active agents. This work demonstrates M+N pipetting steps instead of 2xMxN pipetting steps carried out on bench. This significantly reduces the number of pipetting steps usually carried out on bench by liquid handling robots.

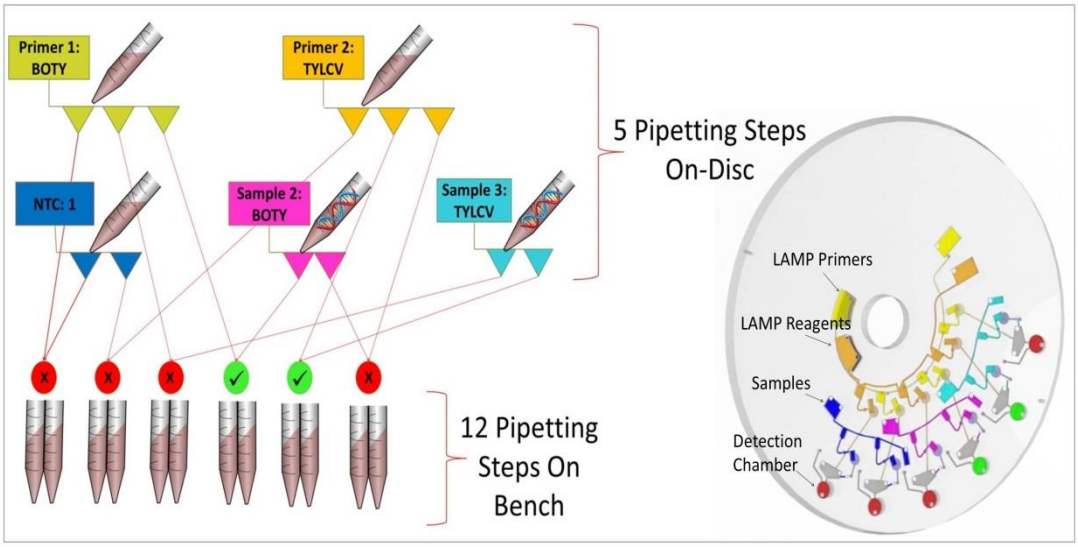

Figure 31: A schematic representing the combinatorial mixing of samples with primers. A significant reduction of pipetting steps on bench compared to pipetting steps required on bench. The samples and primers are colour coded to allow visualisation of their location and transport through the microfluidic disc. The samples are then collected in the detection chamber and expected DNA amplification is represented by the colour green. Red indicates no DNA amplification.

4.6.2 Testing Operation

BOTY and TYLCV DNA samples were tested on this microfluidic disc. As described in Section 3.3.2 there are four components which make up one complete sample reaction. The BOTY primers with the addition of the isothermal mastermix were loaded into the first large primer chamber. The TYLCV primers with the addition of the isothermal mastermix were loaded into the second large primer chamber. A primer stock was prepared for each set of primers with the addition of isothermal mastermix prior to loading on disc which was sufficient for at least twenty-five reactions. All sample preparation was carried out in sterile conditions as outlined in Section 3.3.1. The isothermal mastermix and primer volumes are outlined in Table 10.

Isothermal mastermix and primer stock preparation	
	25x (µL)
Isothermal Mastermix	375
FIP (40 µM)	31.25
BIP (40 µM)	31.25
B3 (40 µM)	3.125
F3 (40 µM)	3.125
LF (40 µM)	18.75
BF (40 µM)	18.75
Total	481.25

Table 10: Isothermal mastermix and primer stock preparation. The required volumes to prepare a 25x isothermal mastermix and primer stock.

The first sample chamber was loaded with an NTC which consisted of 3 % BSA solution. The second sample chamber was loaded with the BOTY DNA sample with 3 % BSA solution and the third sample chamber was loaded with the TYLCV DNA solution sample with 3 % BSA. The volumes loaded into each chamber are outlined in Table 11.

Colour of Chamber	**Volume**	**Components of Volume Loaded**
Yellow	60 µL	BOTY Primers + Isothermal mastermix
Orange	60 µL	TYLCV Primers + Isothermal mastermix
Dark Blue	30 µL	NTC (3 % BSA Solution)
Purple	30 µL	3 µL BOTY DNA Sample + 27 µL 3 % BSA Solution
Light Blue	30 µL	3 µL TYLCV DNA Sample + 27 µL 3 % BSA Solution

Table 11: The components loaded into each chamber on 2x3 microfluidic disc. The volume and break down of components is clearly given. The coloured schematic (Figure 32) is provided as a reference to visualise the location of each component.

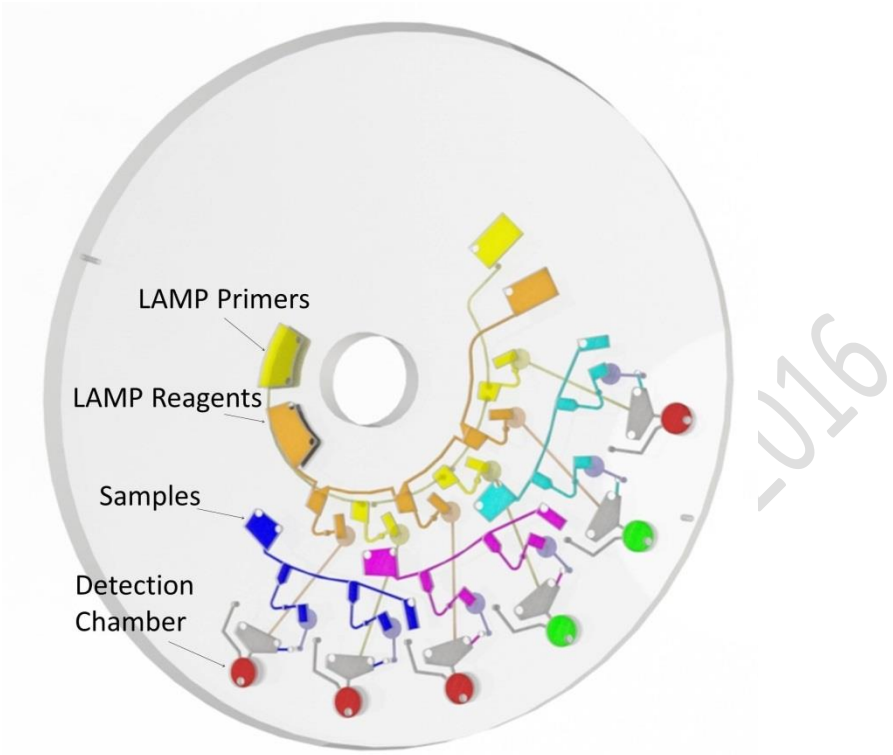

Figure 32: A coloured schematic of 2x3 disc for visual representation (for Table 11) of the location of each component.

The testing was carried out as described in Section 4.3. Once the testing on the spin stand was complete the disc was sealed with one full sheet of clear PSA that covered the complete surface area of the disc. This sealed any vent holes and decreased the possibility of any loss of sample due to evaporation. The disc was wrapped in foil and placed in a standard laboratory oven at 70 ˚C for a total time of 1 hour. The samples were collected from the detection chambers on the disc and loaded onto a gel for amplification confirmation by gel electrophoresis as described in Section 3.3.3.2.

A gel containing no DNA samples and only NTCs was performed for comparison with the addition of a 1 KB ladder. This gel did not show any DNA amplification as expected. DNA amplification was observed from the gel containing the DNA samples (prepared as

described in Section 4.4.2). Long smeared bands (due to the presence of large amounts of DNA) were observed which indicated DNA amplification had occurred.

The results obtained from the gel and observed with the UV imager are as outlined below.

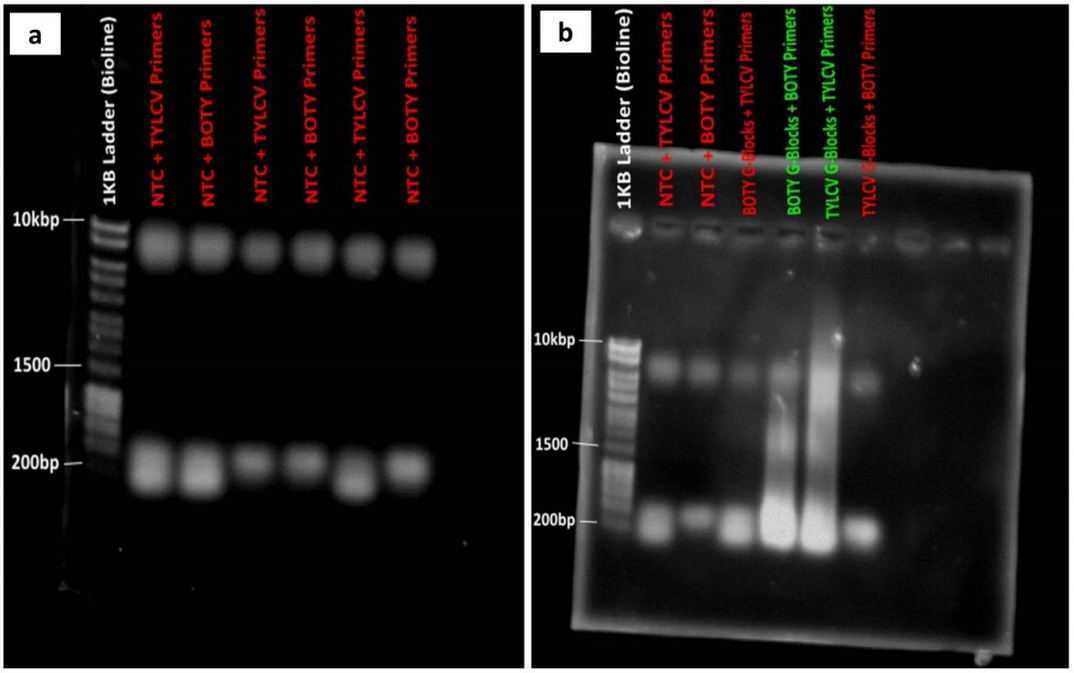

Figure 33: The results obtained from the gel loaded with NTCs and DNA samples. a) A gel loaded with NTCs only to show a negative control. b) A gel loaded with BOTY (DNA sample and primers), TYLCV (DNA sample and primers) and NTCs. The long smeared bands show DNA amplification occurred.

4.7 Discussion

The work described in this chapter was focused on implementing LAMP amplification on Lab-on-a-Disc in a manner which permitted highly-scalable combinatorial mixing. The main aim was designing a microfluidic disc that permitted 2x3 combinatorial mixing of samples and target-specific primers which could be amplified using LAMP on disc.

Initially the disc was designed and fluidically tested using various coloured food dyes. The design was optimised and resulted in a fully functional microfluidic disc. The integration of DF tabs allowed for controlled fluid movement and reagent storage.

The initial testing of samples and target-specific primers on a simple test chip resulted in the failure of amplification. Numerous tests were carried out with no successful amplification. As a result of this failed amplification on chip various investigations were carried out.

Various surface treatments were carried out on small pieces of PMMA which were placed into PCR tubes with sample. The PMMA pieces were treated as follows: untreated PMMA, MICRO90 washed PMMA and MICRO90 washed and BSA blocked PMMA. It was concluded that untreated PMMA had an inhibitory effect on the amplification of samples and no amplification occurred. The pieces of PMMA treated with MICRO90 wash and MICRO90 wash and BSA blocked showed positive amplification indicating that PMMA pre-treatment was necessary for samples to amplify. It was also noted that the PMMA treated with Micro90 wash and BSA blocked showed the closest amplification times to samples in PCR tubes without any PMMA added. This suggested that the addition of BSA to the system did not inhibit amplification and was necessary to obtain positive amplification.

Following on from the surface treatments of small pieces of PMMA the surface treatments were then applied to small test chips. MICRO90 wash was applied to three test chips while MICRO90 wash and BSA blocked was applied to another three test chips. This was carried out to further test the surface treatments on a larger scale. A simple test chip was designed containing eight circular chambers. Various test conditions were applied to each test chip. DI water was loaded into seven chambers and red food dye was loaded into one chamber. This was carried out to determine any sealing issues the surface treatments might produce. After testing it was determined that the test chips surface treated with MICRO90 wash showed no signs of leaking and remained intact while the test chips surface treated with MICRO90 wash and BSA blocked showed significant leaking. It was concluded that the test chips surface treated with MICRO90 wash and BSA blocked showed significant signs of leaking and BSA could not be added into the surface treatment of the disc prior to manufacture. The red food dye was clearly seen in other chambers contained on the test chip demonstrating inefficient sealing. The test chip could also be easily pulled apart by hand. As it was determined that the addition of BSA was necessary for optimum amplification to occur the integration of BSA had to be implemented by another approach.

BSA was then added directly into the sample. A 3 % BSA solution was added to the sample preparation protocol. This concentration of BSA was advised by a colleague with great experience in this area. The nuclease free water normally used in the protocol was substituted with the same volume of 3 % BSA solution. Three samples containing nuclease free water were placed into separate PCR tubes and three samples containing 3 % BSA solution were also placed into separate PCR tubes. The amplification was carried out on the Rotorgene 6000. It was determined that the addition of BSA solution into the sample preparation showed positive amplification. It could be concluded that the addition of BSA directly into the sample preparation had positive effects on the sample and showed no

inhibition of sample amplification and was used for the remainder of testing. It can also be concluded that the addition of the BSA is important for the overall amplification to occur using PMMA in the manufacture of the discs. The option of using other materials to manufacture the microfluidic discs is not suitable as these discs require complex vertical vias and microchannels to transport the various components about the disc. Other materials and disc manufacturing methods cannot provide such complex networking of channels and chambers which are required for the overall operation of the microfluidic system.

The samples and target-specific primers were then tested on disc. The PMMA was surface treated with MICRO90 wash and BSA was added directly into the sample preparation. This resulted in a system that worked and lead to success in combinatorial mixing and sample amplification.

Towards the future this system can be scaled to permit, for example, 10x10 or 30x30 combinatorial mixing on disc. This could be achieved as volumes can be scaled by making reservoirs smaller thus permitting more structures to be placed on disc. The centrifugal force can also be increased which will pump liquid through smaller channels and burst DF valves even if the fluid height is smaller. However, to reach this we would need to address some issues. As there is limited space on disc this would inhibit valving size. As the level of combinatorial mixing increases positioning individual tabs will become extremely difficult to implement reliably and would also be very time consuming. Loading volumes would also increase requiring increased storage of reagents on disc. Issues would also arise with manufacturing methods as the current method is not compatible with miniaturisation. Milling, hot embossing and injection moulding can permit much smaller feature sizes. Fluorescence based detection of LAMP samples amplified on chips manufactured and

cleaned using these protocols was confirmed by an unpublished undergraduate project conducted within our laboratory under my joint technical supervision[103]. Based on these results it is clear that the methods here could be used for real time monitoring of real time amplification. Furthermore, detection systems could be integrated in the DNA amplification process to obtain results in real time. For e.g. a hot air gun could be easily integrated onto the spin stand to heat the rotating disc while obtaining real time results using a fluorescent detection system.

Chapter 5

6x4 Combinatorial Mixing

5.1 General Introduction

Chapter 4 demonstrated a 2x3 combinatorial mixing disc to mix samples with target-specific primers for rapid pathogen detection on a centrifugal platform. This microfluidic disc successfully achieved mixing of two different target-specific primer sets with three different samples to create a combinatorial mixing system. This chapter demonstrates, using a similar approach, a 6x4 combinatorial mixing disc. This generates twenty-four unique outputs compared to the six unique reaction outputs created by the 2x3 microfluidic disc. This is a progressive step towards a highly scalable combinatorial mixing microfluidic system.

In the 2x3 disc, the isothermal mix was pre-mixed with the target-specific primers to create a reaction mixture from two components. This 6x4 disc is designed to create the reaction mixture from the three major components; sample, primers and LAMP reagent. In view of this, along with 6x4 mixing of sample and primers, LAMP reagent is loaded into a sample reservoir and metered into twenty-four aliquots. Similarly, to prevent evaporation, it was identified that mineral oil must also be metered and distributed to all twenty-four reaction wells. Thus, through twelve loading operations (six of target-specific primers, four of the sample, one of LAMP reagent and one of mineral oil) this disc is designed to create assays which otherwise would require ninety-six pipetting steps. To create the high density of microfluidic structures needed for this approach, the disc architecture was extended

through multiple layers. This chapter describes the manufacture, operation and the fluidic capabilities of this 6x4 combinatorial mixing system. On-disc amplification LAMP is not demonstrated due to time constraints and so going forward with microfluidic analysis various food dyes were used to demonstrate fluidic viability.

5.2 Disc Manufacture and Assembly

This microfluidic disc (160 Ø mm) was manufactured and assembled as described by the methods in Section 3.1.2-3.1.4. Due to the greater structure density required for 6x4 mixing, the disc architecture was extended across twelve different layers of PMMA, PSA and DF. The manufacturing process differs from our standard techniques (i.e. as used for the 2x3 microfluidic disc) in two major ways. Firstly, the layers coloured orange Figure 34 were manufactured by sticking the PSA sheet directly onto the PMMA layer prior to structuring by laser ablation. This was performed as the PSA and PMMA layer were identical (in design and size) and this allowed a reduction in manufacturing time as two processes were carried out at one time. This approach also increases reliability as the two layers are, by definition, perfectly aligned.

In the second variation from the standard manufacturing practice, an entire sheet of DF was placed across the disc rather than individually placed ninety-six DF tabs; an approach which would have been unreliable (due to potential human error during manufacturing) and time consuming. KC35 film was used as the DF as it has a dissolution time of 30 seconds [88]. The sheet of DF, layer 9 in Figure 34 was placed between layers 8 and 10 and secured by PSA. The sheet of DF was assembled in the disc as described in Section 3.1.4.2. The use of KC film and the novel valving system will be discussed in Section 5.2.2.

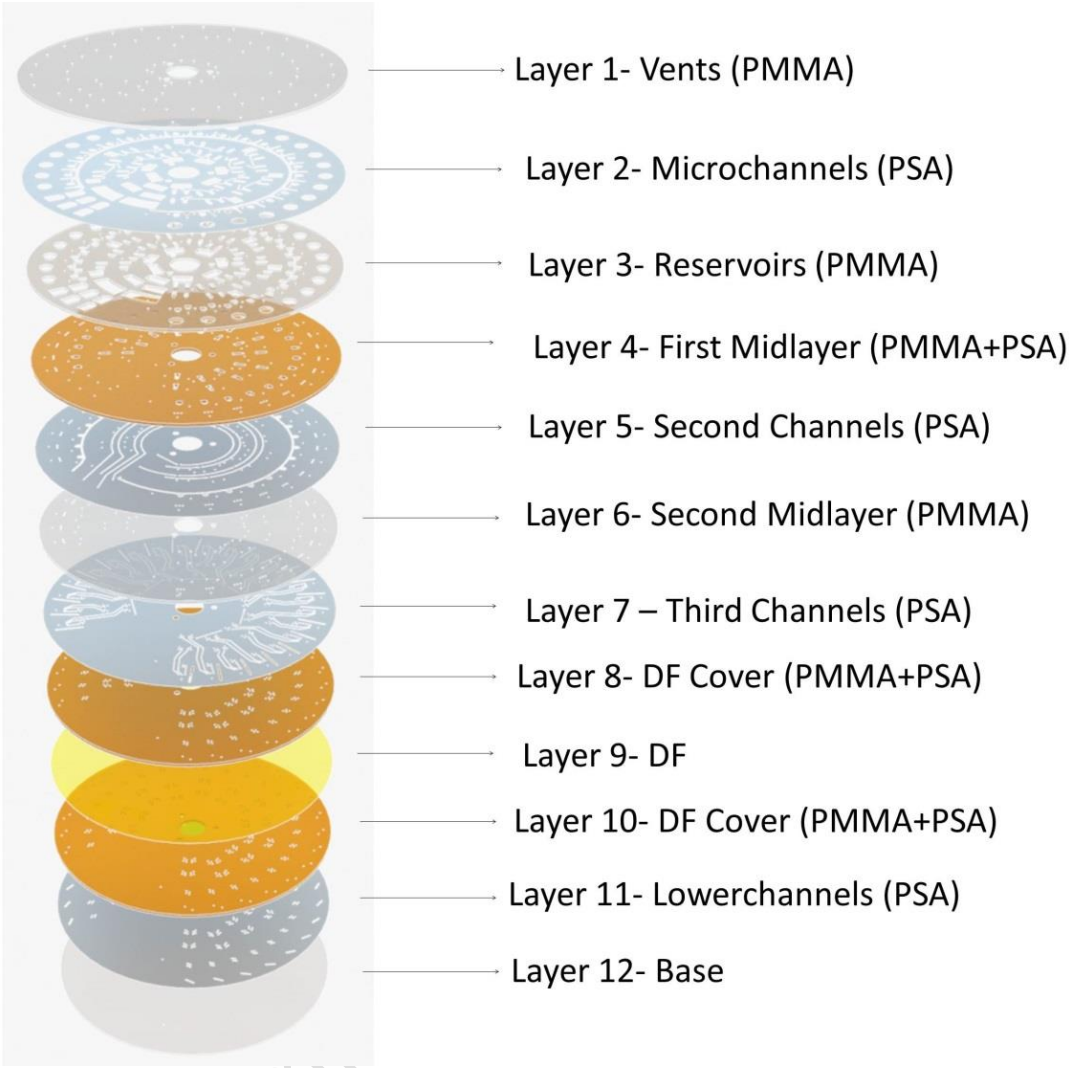

Figure 34: Detail of 6x4 disc assembly. A total of twelve layers included in the disc assembly which allowed a complex 3-dimensional multi-layer structure.

The purpose of each layer is described in detail in Section 3.1.4. The addition of extra mid-layers and channels allowed for more complex liquid transport around the disc. Microchannels are defined in layers 2 and layer 5 and are oriented such the channels in layer 2 travel around the disc while the channels in layer 5 travel radially outwards from the centre. This crossing architecture enables the MXN combinatorial mixing. Microchannels in layer 2 also allowed LAMP reagent and mineral oil to be metered and aliquoted into

twenty-four different reservoirs. Microchannels in layer 7 are oriented to transfer metered liquid radially outward to the mixing chambers located on the periphery of the disc. Voids in the PMMA layers form metering chambers also act as vertical vias which permit the liquid to move from one microchannel to another.

Various coloured food dyes were used for the microfluidic testing of this disc (shown in Figure 35). The dyes travelled from layer 7 to layer 9 where the KC film was positioned. The liquid dissolved and flowed through the KC film and travelled upwards towards layer 7. As this microfluidic disc is manufactured using complex multiple layers the vertical vias allow for fluid to flow in a downwards and upwards through the microchannels. This occurred multiple times passively dissolving the DF tab. The addition of extra channel layers was required to successfully meter the mineral oil throughout the microfluidic disc. Due to the addition of extra chambers on this disc multiple layers were required to make this system fluidically possible.

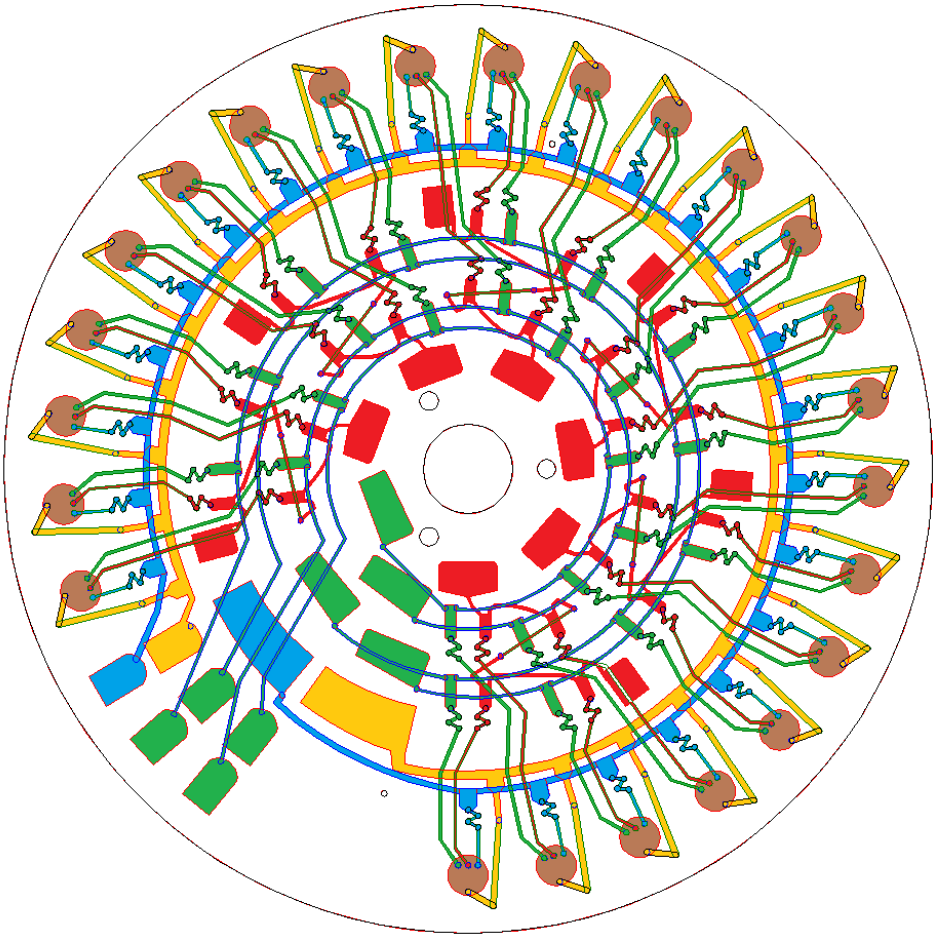

Figure 35: A Coloured schematic highlighting the liquid pathways of various components on 6x4 disc. Layer 2 (yellow) allows for complete metering of the mineral oil. Layer 5 (blue) permits metering of the isothermal mastermix and metering of sample components. Layer 7 (red) permits radial metering of target-specific primers on disc. All components are collected in the detection chamber located at the periphery of the disc (brown).

A second vent layer was added into the manufacture of the disc to allow additional fluid volume in the sample, primer, isothermal mastermix and mineral oil chambers. This was positioned under layer 1 (vents) and held in place by clear PSA to allow the microfluidic channels to be seen. This additional vent layer acted as a second reservoir to create extra loading volume for the sample, target-specific primer, isothermal mastermix and mineral oil chambers.

The PMMA thickness used for each PMMA layer is provided in the table below.

PMMA thickness in disc manufacture			
PMMA (mm)	0.3	1.5	2
Layer no.	1,4,6,8,10,12	3	Second vent layer

Table 12: The various PMMA thickness used in the manufacture of the 6x4 microfluidic disc.

Each PMMA layer was fabricated using the laser machine and cutting parameters as described in Section 3.1.2.1. Table 1.

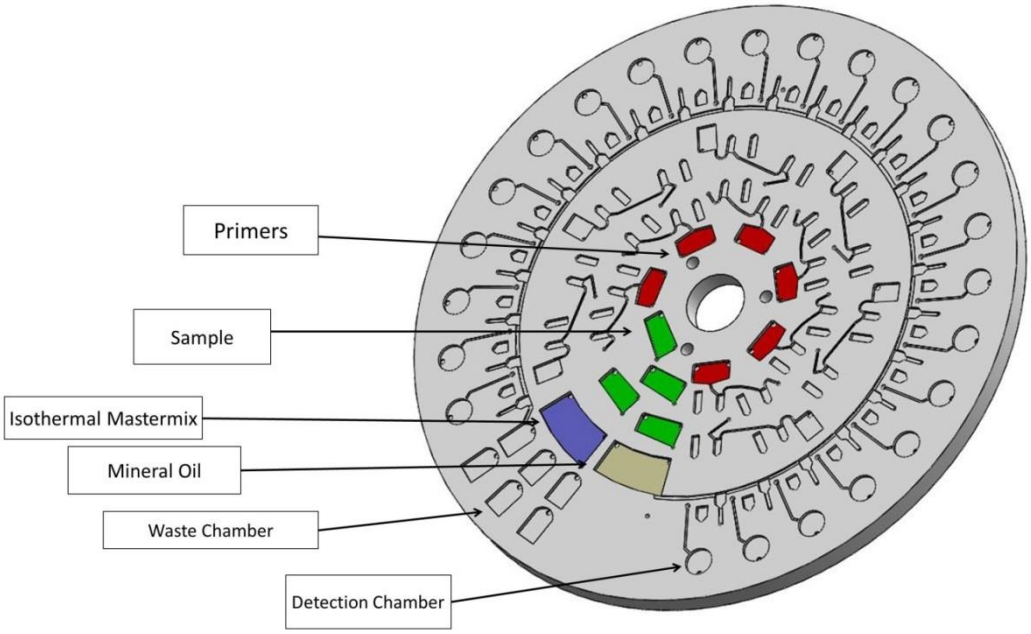

Figure 36: Labelled schematic of the 6x4 microfluidic disc. The key structures and features are clearly labelled. The six inner primer chambers (red) meter into four separate aliquots each. The four sample chambers (green) meter into six separate aliquots each. The isothermal mastermix (blue) and mineral oil (yellow) meter into twenty-four separate aliquots each. Waste chambers are located at the end of each loading chamber to retain any excess liquid on the disc.

5.2.1 Working Principle of Valving System

This 6x4 microfluidic disc demonstrates a novel valving system whereby a number of DF tabs are positioned in sequence resulting in a time delay reaction to release components. This time delay sequence allows for all components to meter fully around the entirety of the disc before being collected in the detection chamber. The novel valving system used on this disc uses one whole sheet of DF cut to size of the disc. As mentioned in Section 5.2.1. this is carried out to reliably position the equivalent of ninety-six DF tabs.

First, the mineral oil is metered to each respective channel (shown in Figure 37). This travels downward and is stopped from entering the detection chamber by the position of a DF plug. The DF does not dissolve upon contact with mineral oil and so the mineral oil is stored until needed. Second, the isothermal mastermix, samples and target-specific primers are metered. As they are metered around the disc each component will come into contact with the first DF tab. This will take 30 seconds to dissolve until the component is allowed to travel to the next DF tab where it will take a further 30 seconds to dissolve. In total there are four DF tabs positioned in sequence to allow a 2-minute time delay. This permits each component to fully meter around the entirety of the disc before being collected in the detection chamber. As each component dissolves the fourth DF tab they are collected in the detection chamber and mixed. Positioned at the bottom of the detection is another DF tab. This dissolves upon contact with the components and allows the mineral oil DF plug to dissolve. The mineral oil is then released and travels upward to the detection chamber where it acts as a barrier to avoid loss of sample by evaporation.

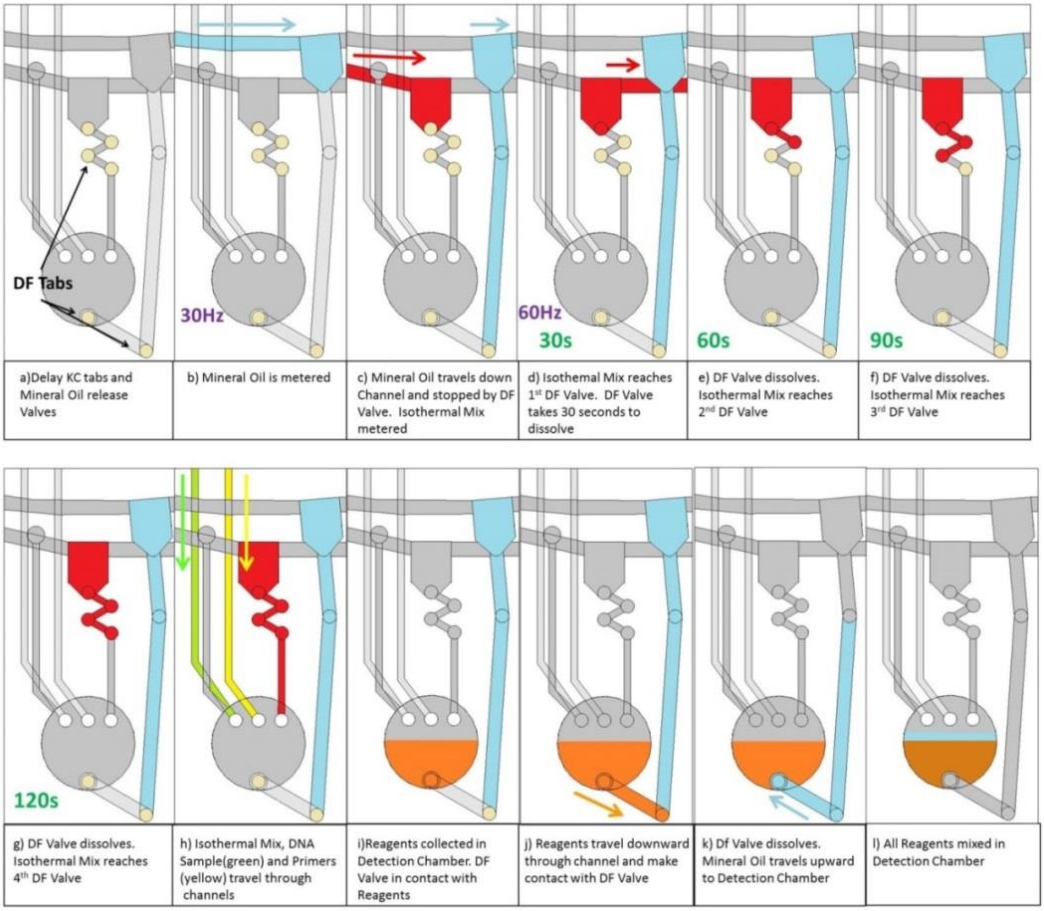

Figure 37: A schematic of the novel valving system in 6x4 disc. a) Delay DF tabs and mineral oil release tabs are highlighted in yellow. b) The mineral oil is metered (blue) at 30 Hz. c) Mineral oil travels down the channel and is stopped by the DF tab. The isothermal mastermix is metered (red at 60 Hz). d) The isothermal mastermix reaches the first DF tab. e) The DF tab dissolves after 30 seconds and the isothermal mastermix continues to travel downward to the second DF tab. f) The DF tab dissolves after a further 30 seconds and the isothermal mastermix travels downward to the third DF tab. g) The DF tab dissolves after a further 30 seconds and the isothermal mastermix travels downward to the fourth and final DF tab. h) Isothermal mastermix, DNA sample (green) and primers (yellow) travels downward through channels. i) Reagents collected and mixed (orange) and wet the DF tab located at the bottom of the detection chamber. j) Mixed reagents travel downward through channel and wet DF tab isolating mineral oil. k) DF tab dissolves and the mineral oil is allowed to travel upward towards the detection chamber. l) The mineral oil forms a layer on the surface of the mixed reagents to produce an air-tight chamber.

5.3 Operation of Disc

The disc contained four sample chambers, six target- specific primer chambers, one isothermal mastermix chamber and one mineral oil chamber.

Different coloured food dyes were used as a visual guide to fluidically test the system. The disc was mounted to the spindle motor on the spin stand and securely fit with a screw. The PCO and LABview software were initialised as described in Section 3.2. Multiple tests applying various spin frequencies were carried out to determine the optimum spin frequencies for the disc operation. The final spin frequency protocol was determined. First, 300 µL of mineral oil was loaded onto the disc. The initial spin frequency to allow the complete metering of mineral oil was performed at 40 Hz (shown in Figure 39(a)). Each small metering chamber was designed to hold 13.5 µL. The disc was stopped once the mineral oil metered fully. The sample, primer and Isothermal mastermix chambers were filled while the disc was stopped and mounted on the spin stand. The six primer chambers (red) were each filled with 130 µL of red food dye and each of their smaller metering chambers could hold 12 µL. The four sample chambers (green) were each filled with 180 µL of green food dye and each of their smaller metering chambers could hold 12 µL. The isothermal mastermix chamber (blue) was filled with 300 µL of blue food dye and the smaller metering chambers could hold 13.5 µL. When all of the chambers were loaded the spin frequency was set to 30 Hz and the spindle motor started again. The red, green and blue food dyes were metered successfully at 30 Hz. When metering had completed the spin frequency was increased to 60 Hz to allow the DF valve to completely dissolve and allow the various coloured food dyes to be collected in the detection chambers and to allow the release of the stored mineral oil.

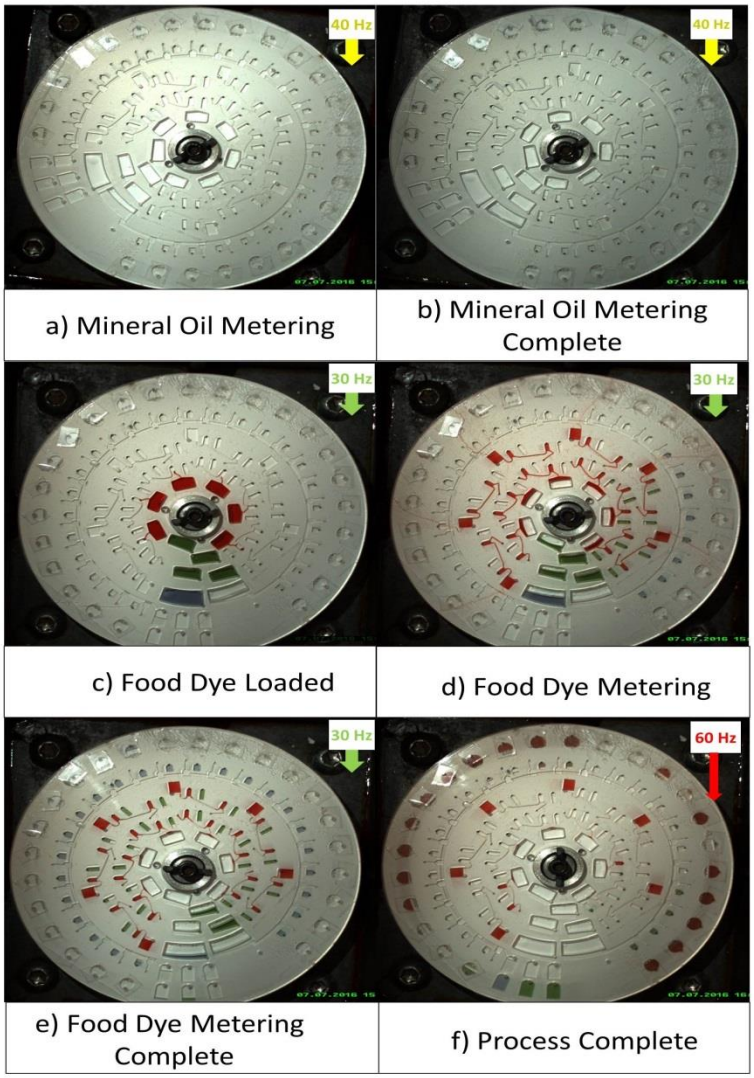

Figure 38: Fluidic testing of 6x4 microfluidic disc. Coloured food dye is used for visual guide; red- target- specific primers, green- samples, blue- Isothermal mastermix, clear liquid- mineral oil.(a) Mineral oil is loaded on disc and spun at a frequency of 40 Hz. (b)Metering of mineral oil is complete and disc is stopped to load coloured food dyes (c) Each sample (sample 1 to M) loaded on the disc is divided into 'N' aliquots while each of the primers (primers 1 to N) loaded onto the disc is divided into 'M' aliquots. (d-e) Once metering is completed, the disc spin rate is increased to open the DF burst valves. (f)The liquid elements are then pumped, through a multi-layer architecture, to reservoirs on the periphery of the disc where each individual sample mixes with an individual reagent to create twenty-four MXN unique reagent combinations.

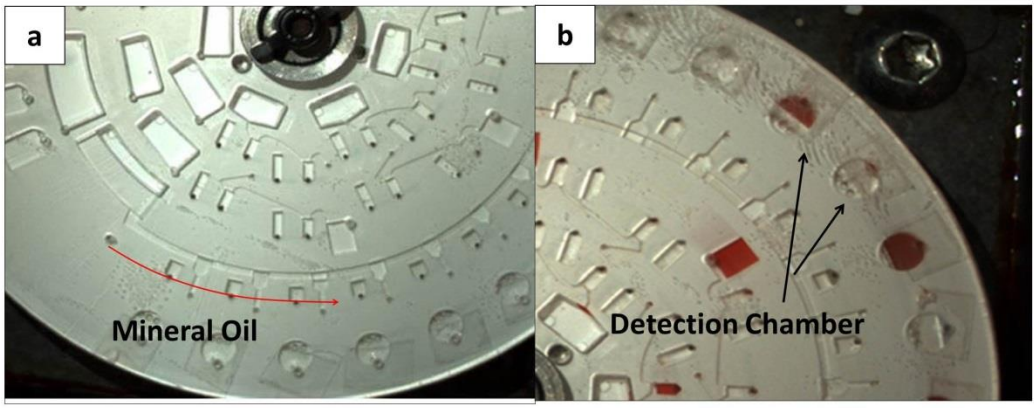

Figure 39: 6x4 zoomed in images. a) Shows the metering of the mineral oil in the direction of the red arrow. The small metering chambers are being filled with mineral oil at 40 Hz. b) The leaking of the disc is clearly shown. The food dye has been collected in some detections chambers and others are empty. This indicates the food dye has travelled to other locations on the disc.

5.4 Discussion

This 6x4 microfluidic disc demonstrates the capabilities of a highly scalable combinatorial mixing microfluidic system. The generation of twenty-four unique sample/reagent outputs demonstrates a highly automated system for highly scalable combinatorial mixing. The multi-layer architecture permits complex liquid transportation around the entirety of the disc. The design allowing all components to be loaded individually increases reliability and is intended to reduce cross contamination between components stored on the disc. A significant reduction of pipetting steps was also demonstrated where ninety-six pipetting steps would have been required on bench compared to twelve demonstrated on disc. The novel valving system, using one whole sheet of DF, as opposed to the equivalent of ninety-six individual DF tabs used in Chapter 4, increases the reliability of fluid control and component storage. This unique time sequence delay ensures all components are fully metered around the entirety of the disc and are isolated before being collected in the detection chamber.

However, although there have been major advances through numerous fluidic testing of this design with various coloured food dyes adjustments are required to improve its reliability. As metering of all components has been achieved (as seen by the successful metering of the red, blue and green food dyes around the disc) successful collection of all components in the detection chamber has not been fully achieved. Most components have been successfully collected in their designated detection chamber with the exception of a few travelling to other locations on the disc (shown in

Figure *38* (f)). As seen in Figure 39(b) some of the detection chambers remain empty or show only a single food dye colour which suggests very minor leaking in some areas of the disc as the food dyes have travelled to other locations on the disc. This minor leaking could be a result of a design flaw or from inefficient sealing during the manufacture and assembly of the disc. If in fact LAMP reagents and samples were tested on this disc instead of food dyes cross contamination would be have been likely to occur. This can be easily rectified by carefully adjusting the disc design. The chamber volumes also require adjustments as the isothermal mastermix chamber is slightly below the optimum component volume.

This 6x4 microfluidic disc is very close to functioning fluidically which can then be used for LAMP detection analysis. This disc shows great potential to successfully carry out combinatorial mixing with an output of twenty-four unique sample/reagent combinations in an autonomous manner.

Chapter 6

10x10 Combinatorial Mixing

6.1 General Introduction

Chapter 5 demonstrated a 6x4 combinatorial mixing disc intended to mix six target-specific primers with four samples generating twenty-four unique sample/reagent outputs. This disc successfully achieved the complete metering of all components to their respective metering chambers. This chapter demonstrates a disc with the same principle of mixing 'M' samples with 'N' reagents to produce one hundred unique sample/reagent outputs in an autonomous manner. This disc is designed to permit twenty loading operations (ten sample and ten target-specific primers with isothermal mastermix) to create assays which would otherwise require two hundred pipetting steps. This chapter describes the manufacture, operation and the fluidic capabilities of this 10x10 combinatorial mixing system. On-disc amplification LAMP is not demonstrated due to time constraints.

6.2 Disc manufacture and assembly

This microfluidic disc was manufactured and assembled as described by the methods in Section 3.1.2-3.1.4. The disc architecture was extended across eleven different layers of PMMA, PSA and DF. The manufacturing process follows the same process as described in Section 5.2.1. The layers coloured red (layers 3, 5, 7, 9) (shown in

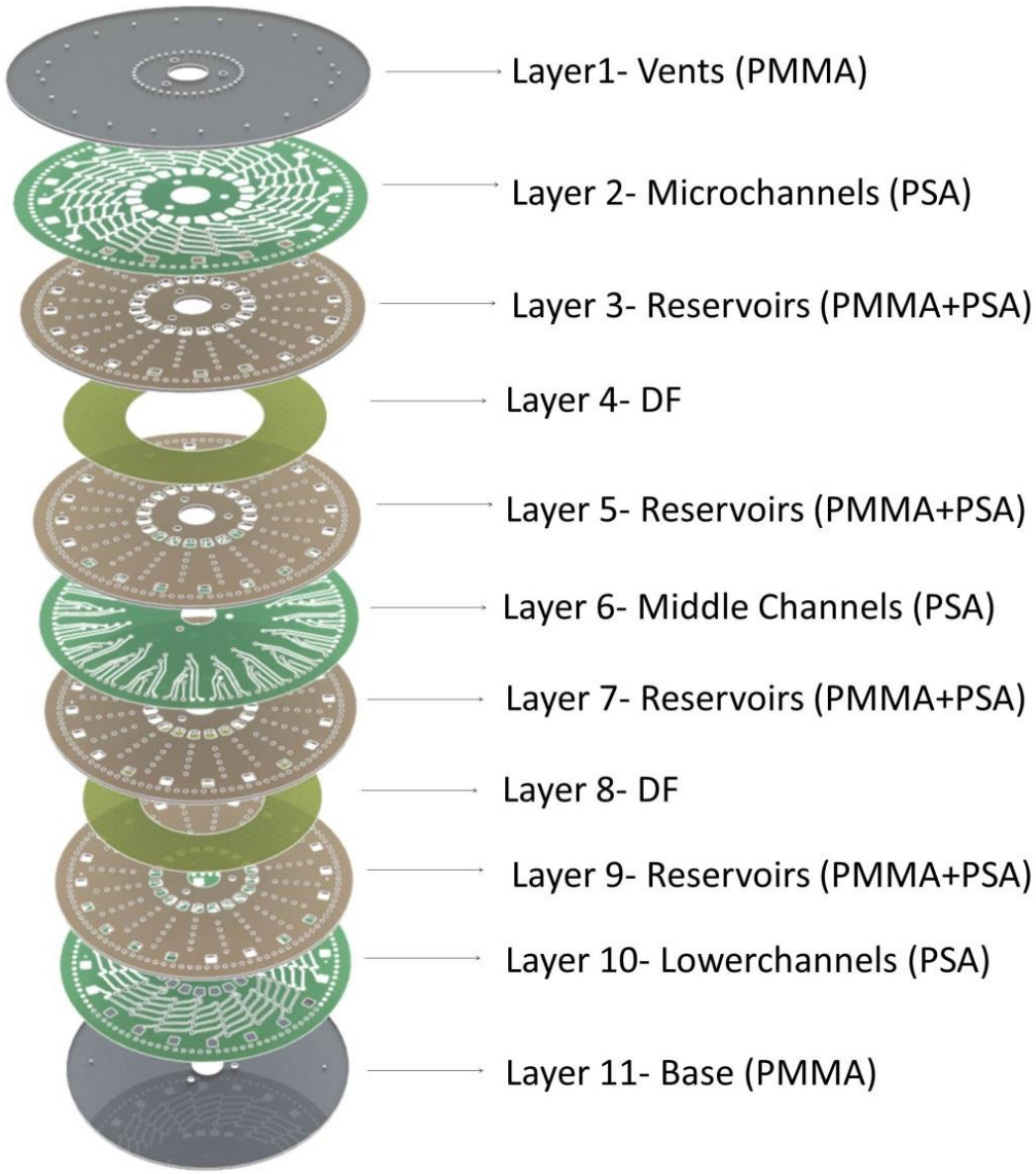

Figure *40*) were manufactured by sticking the PSA sheet directly onto the PMMA layer prior to structuring by laser ablation. This was performed as the PSA and PMMA layer were identical and this allowed a reduction in manufacturing time as two processes were carried out at one time. This approach also increases reliability as the two layers are, by definition, perfectly aligned.

This disc differs slightly from the manufacturing process described in Section 5.2.1 as two sheets of DF are placed across the disc rather than individually placed one hundred DF tabs. KC35 film was used as the DF as it has a dissolution time of 30 seconds [88]. Each sheet of

DF was cut to size allowing only the mixing chambers to be covered. This allowed component separation until the DF dissolved to permit mixing. The two DF sheets were positioned between layers 3 and 5; and also between layers 7 and 9 and secured in place by PSA. The sheet of DF was assembled in the disc as described in Section 3.1.4.2. The novel valving system will be discussed in Section 6.2.2.

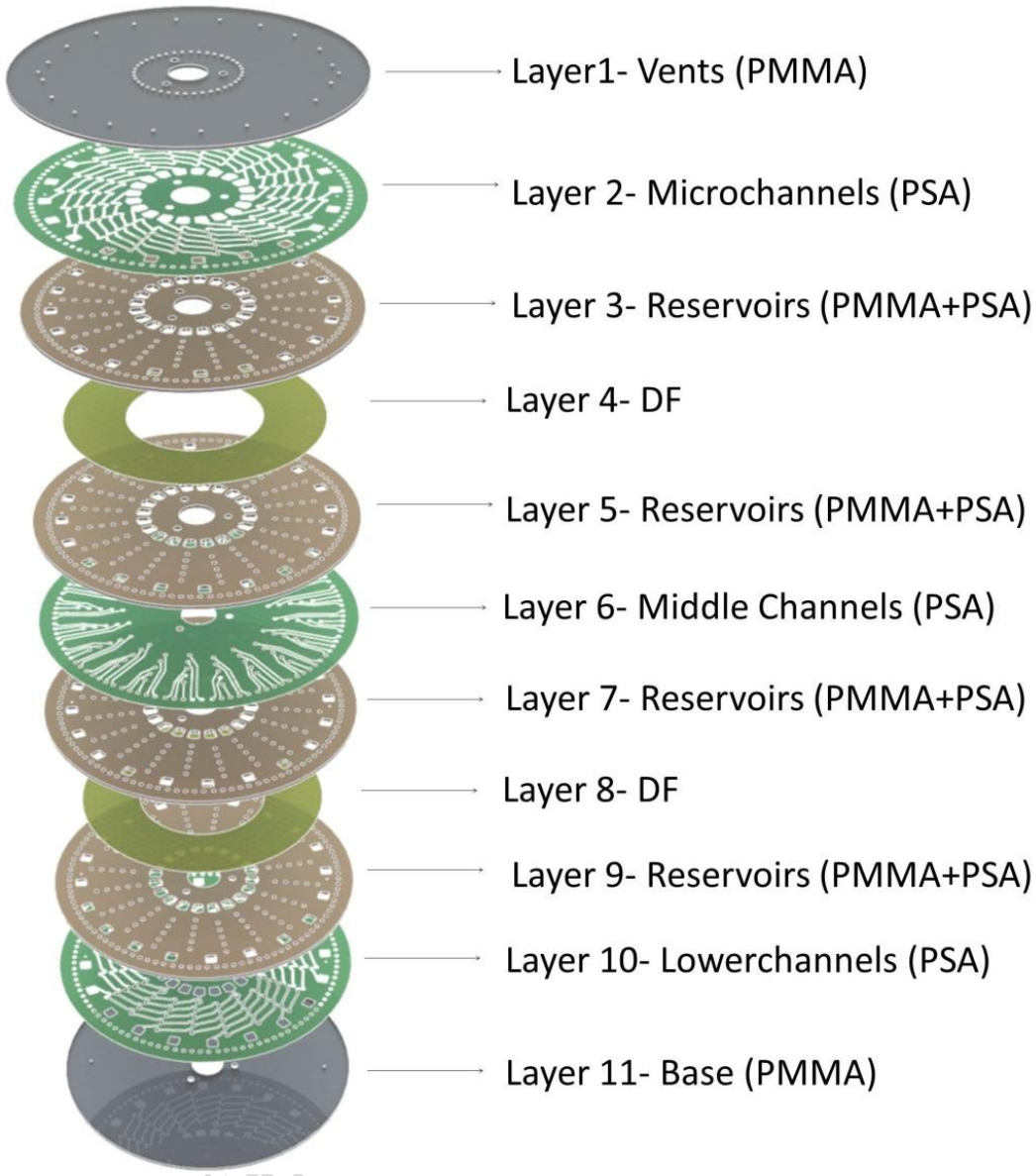

Figure 40: Detail of 10x10 disc assembly. A total of 11 layers included in the disc assembly with 2 sheets of DF allowing a complex 3-dimensional multi-layer structure. The two sheets of DF are carefully placed between layers 3 and 5 and between layers 7 and 8.

The purpose of each layer is described in detail in Section 3.1.4. Microchannels are defined in layers 2 and 10 allowing the sample and target-specific primers to meter respectively. The sample is metered on layer 2 and the target-specific primers are metered on layer 10 which can be seen on the reverse side of the disc. This crossing architecture enables the

MXN combinatorial mixing. The addition of middle channels permits the mixed components to be transported radially outward to the periphery of the disc to be further analysed.

The reservoirs layers (layers 5, 7, 9) were manufactured using 0.3 mm PMMA while the reservoir layer (layer 3) was manufactured using 1.5 mm PMMA. This provided the volume depth required for the loaded components. Various thicknesses of PMMA were used in the manufacture of this disc and are outline in the table below.

PMMA thickness in disc manufacture		
PMMA (mm)	0.3	1.5
Layer no.	1, 5, 7, 9	3, 11

Table 13: The various PMMA thickness used in the manufacture of the 10x10 microfluidic disc.

Each PMMA layer was fabricated using the laser machine and cutting parameters were applied as described in Section 3.1.2.1.

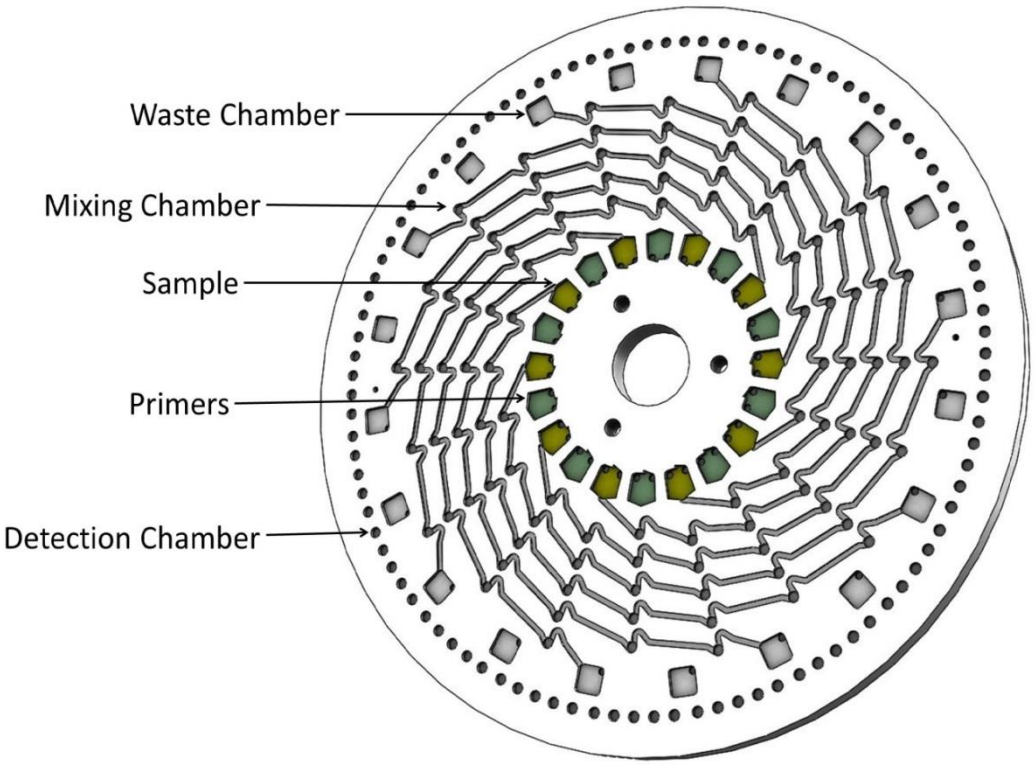

Figure 41: Schematic of the 10x10 microfluidic disc. The sample and primer chambers are located at the centre of the disc. The samples and primers are metered radially outward from these chambers towards the detection chambers located on the outer edge of the disc. Mixing chambers are located at the intersection of the sample and primer channels allowing each component to mix.

6.2.1 Working Principle of Valving System

This novel valving system includes two whole sheets of DF which is placed over the mixing chambers only. All other chambers and features are free from any DF. The positioning of the DF is important as it restricts the sample (yellow) and target-specific primers (green) from prematurely mixing in the mixing chambers. As discussed in Section 6.2 the sample is metered on layer 2 (on top of the disc) and the target-specific primers are metered on layer

10 (bottom of disc). The two DF sheets isolate each component from the mixing chamber until metering has been fully completed. After 30 seconds the sections in contact with each liquid dissolves and each component meet in the mixing chamber where mixing can occur. The mixed components are then transported radially outward through layer 6 and collected in the detection chambers for further analysis

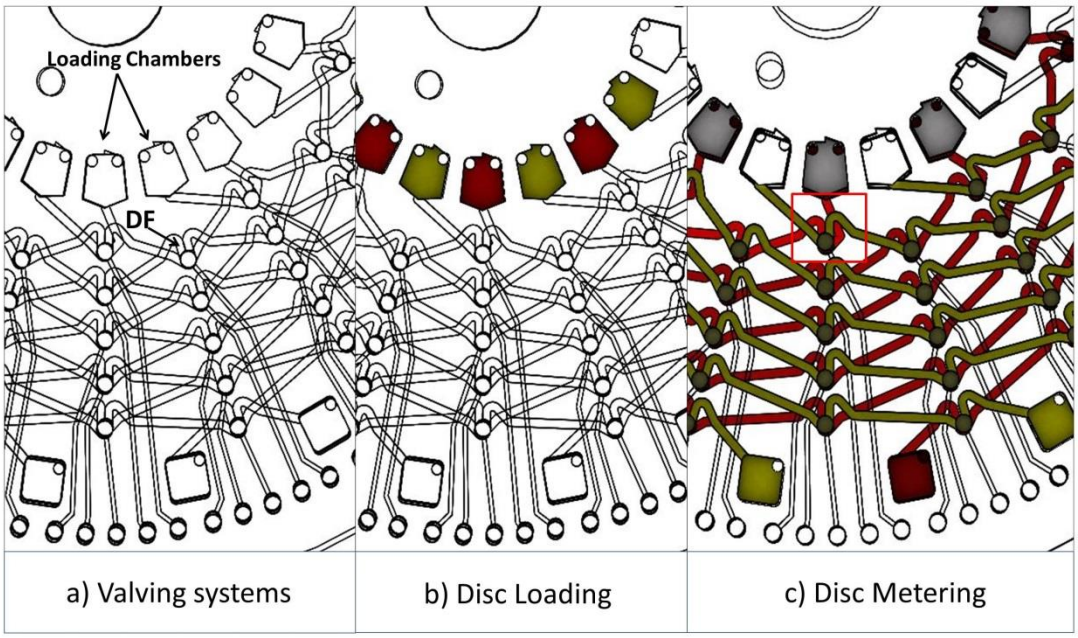

Figure 42: Section of 10x10 disc. A section of the 10x10 mixing disc showing the valving systems. a) The loading chambers and DF are clearly marked. The upper and lower channels form the network involved in the valving system. b) The disc is loaded with three different components (red and yellow) at a spin frequency of 60 Hz. c) The two components are metered around the disc coming into contact with the DFs. The area highlighted in red will be discussed in

Figure 43.

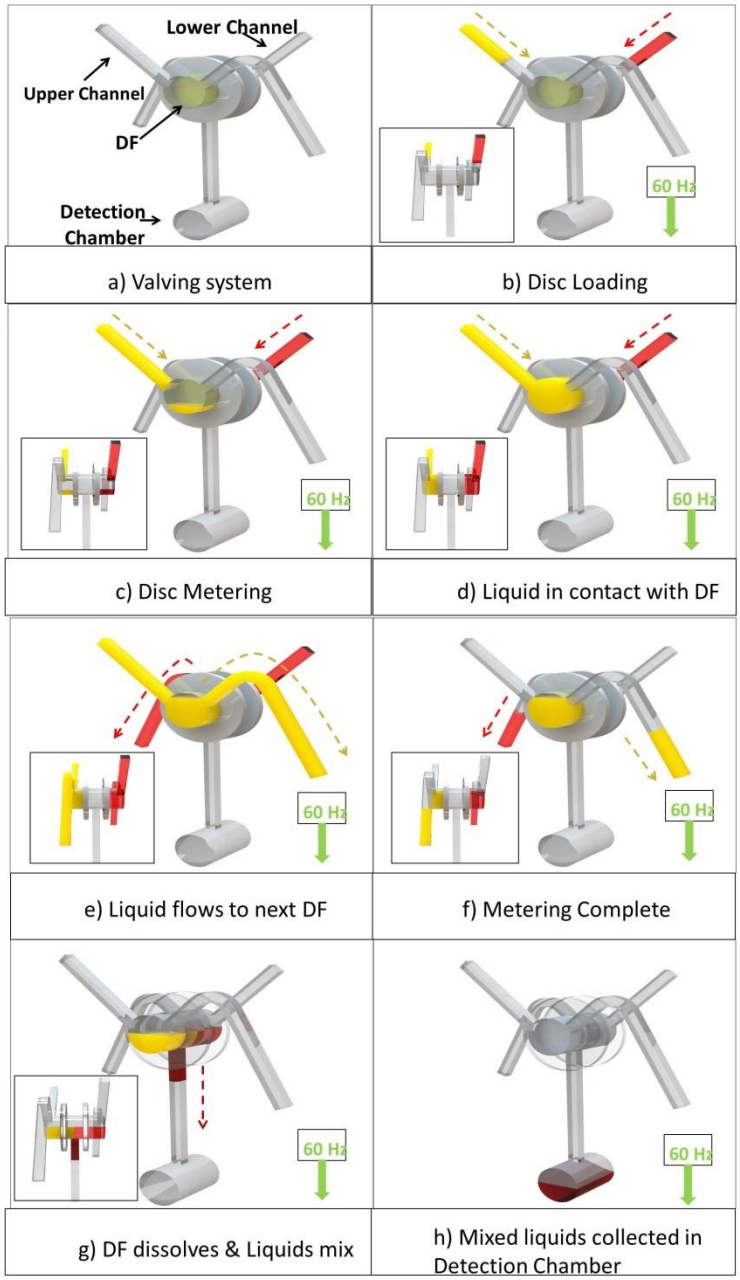

Figure 43: Schematic of working principle of valving system in 10x10 disc with the DF shown in yellow. a) The valving system is shown. b) Sample (yellow) and target-specific primers (red) are loaded into their respective loading chambers. c) Disc is metered at a spin frequency of 60 Hz. d) The chamber is filled and liquids come into contact with the DF. e) Liquids overflow into next chamber by transport channels. f) Metering is complete. g) After 30 seconds the DFs dissolve and the two liquids come into contact and mixing occurs. h) The mixed liquids drop downwards into the detection chamber.

6. 3 Operation of Disc

This disc was fluidically tested using iron nitrate ($FE(NO_3)_3$) and potassium thiocyanate (KSCN). These two components were used as they produce a blood red colour when mixed which would indicate efficient mixing in this microfluidic system. The disc was mounted to the spindle motor on the spin stand and securely fit with a screw. The PCO and LABview software were initialised as described in Section 3.2. Multiple tests applying various spin frequencies were carried out to determine the optimum spin frequencies for the disc operation. The final spin frequency protocol was determined at 60 Hz for the whole duration of testing. The sample and primer chambers were filled with 80 µL of $FE(NO_3)_3$ and KSCN respectively. Each component was metered at a spin frequency of 60 Hz. After 30 seconds each isolated component was released and mixing occurred which was indicated by a change in colour to blood red. The mixture was then transported radially outward toward the periphery of the disc for further analysis. A schematic of the disc operation is shown in Figure 44 and the disc operation is shown in real frames in Figure 45.

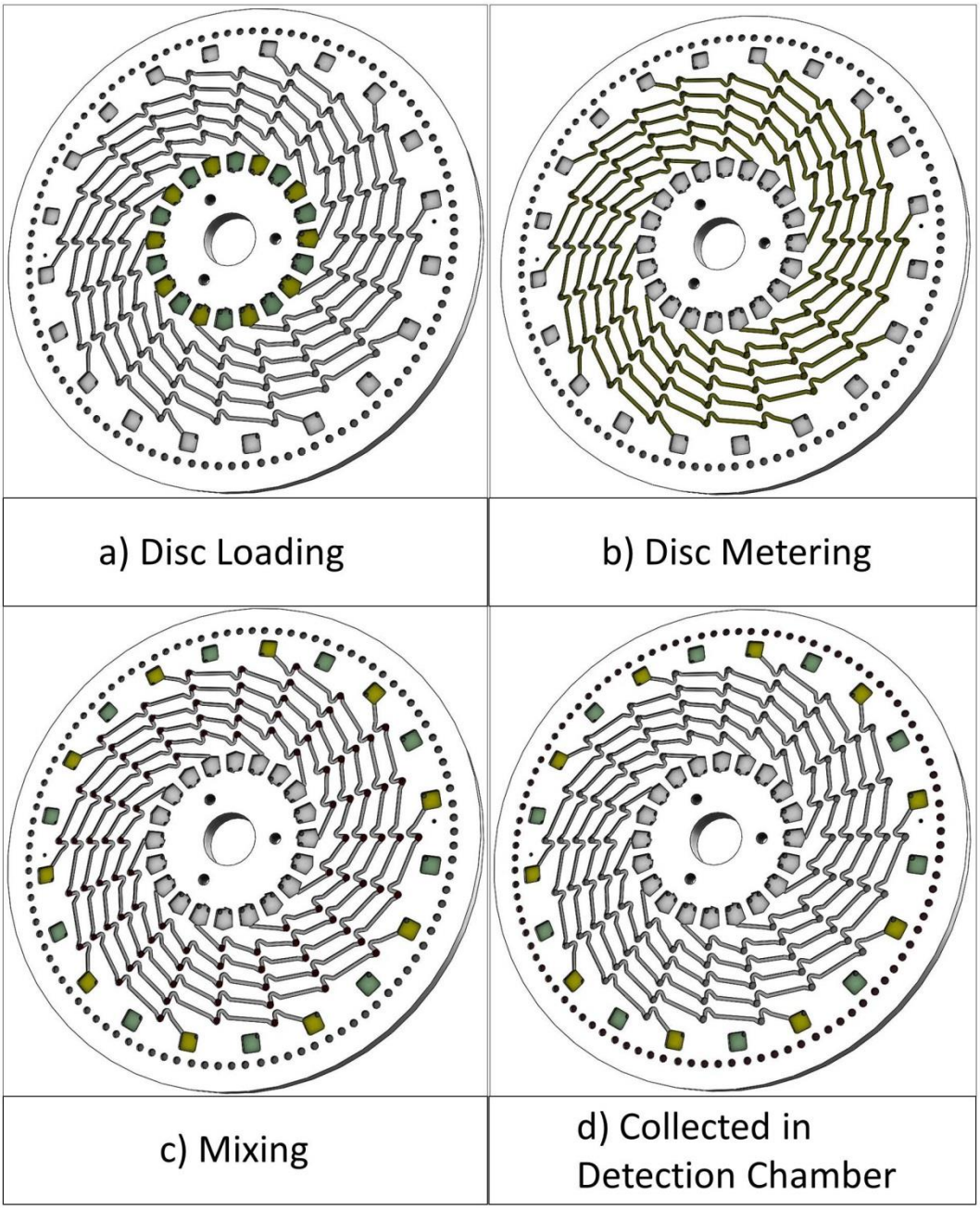

Figure 44: A schematic of the working principle of the 10x10 disc. a) The sample (yellow) and target-specific primers (green) are loaded to their respective chambers. b) Metering is performed at 60 Hz. The sample can be seen metering on the top of the disc as the metering of the target-specific primers is performed on the bottom of the disc. c) After 30 seconds the DF layers dissolve and each component is mixed in the mixing chamber (red). d) The mixed components are transported radially outward to the detection chambers on the edge of the disc.

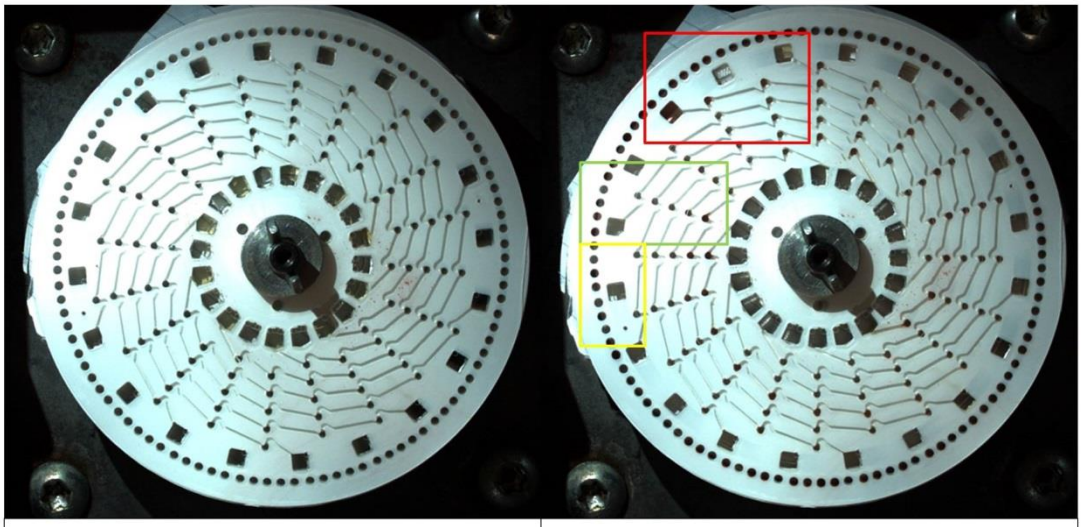

Figure 45: Fluidic testing of 10x10 microfluidic disc. $FE(NO_3)_3$ and KSCN are loaded and metered at 60 Hz. After 30 seconds the DF dissolved allowing each isolated component to mix in the mixing chambers and was transported to the detection chambers on the edge of the disc for further analysis. The areas highlighted red, green and yellow will be discussed further in Figure 46.

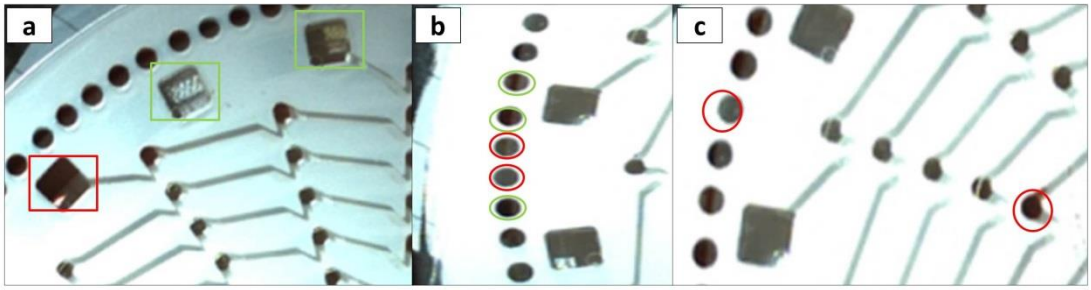

Figure 46: Selected areas on 10x10 mixing disc. a) The green areas show successful metering as the two components remained separate reaching the waste chambers. The red area shows unwanted mixing of the two components (red liquid) and demonstrated premature mixing. b) The green areas contain red liquid and demonstrated successful mixing and collection in detection chamber. The red areas show no collection of red liquid which suggests leaking or the liquid was not released from a mixing chamber. c) The red areas highlighted are two corresponding chambers. It is clearly shown that no red liquid was collected in the detection chamber as it was still trapped in the mixing chamber.

6.4 Discussion

This 10x10 microfluidic disc demonstrates the capabilities of a highly scalable combinatorial mixing microfluidic system. A significant reduction in pipetting steps is demonstrated where a total of twenty pipetting steps generates one hundred unique sample/reagent outputs which is equivalent to two hundred pipetting steps on bench. This disc is easily manufactured and can be scaled to permit higher levels of combinatorial mixing. This design is also more suitable for miniaturisation and shows great potential in doing so. The addition of two sheets of DF improves on the performance as they can be reliably positioned and integrated into the manufacture of the disc. Individually placing DF tabs would be very time consuming and would be unreliable by hand.

However, this disc is showed potential to work but did not function fluidically. Firstly, issues present themselves during the metering process. Some premature blood red liquid could be seen before mixing had occurred which demonstrated the two components were not isolated reliably. This suggested that the DFs prematurely dissolved at approx. 15 seconds whereas KC dissolves at approx. 30 seconds. The primary reason the DFs dissolved early in each case as KC dissolves in approx. 30 seconds under normal centrifugation but fluidic pressure was applied during metering which resulted in it being dissolved sooner (approx. 15 seconds) which was observed during metering. Secondly, upon stopping the disc after testing the mixed components travelled back up the metering channels. This could cause problems with biological sample testing in later stages as cross contamination could occur as a result.

This microfluidic disc shows great potential to develop into a highly scalable combinatorial mixing disc to permit the generation of one hundred unique assay outputs. The disc design

should be reviewed and adjusted to improve the component storage and metering providing a more reliable system.

Chapter 7

Conclusion & Future Work

7.1 Conclusions

This work demonstrated the development of three different microfluidic platforms for the combinatorial mixing of samples (M) with a number of active agents (N). The development of microfluidic devices for pathogen detection is ever increasing as such devices can be manufactured to test for a wide range of pathogenic contaminants using cost-effective materials and can provide rapid answers for point-of-use detection.

7.1.1 2x3 Combinatorial Mixing Disc

The outcomes of the 2x3 combinatorial mixing disc are as follows.

- A disc design for successful 2x3 combinatorial mixing was achieved
- Numerous fluidic tests allowed for optimisation od disc design resulting in a fully functional fluidic system
- Various surface treatments concluded that pre-treatment of PMMA with MICRO90 wash was necessary prior to disc manufacture
- The addition of BSA solution directly to sample preparation was necessary to achieve amplification on disc
- The integration of DF tabs permitted fluid control and reagent storage
- Generation of six unique sample/reagent outputs

7.1.2 6x4 Combinatorial Mixing Disc

The outcomes of the 6x4 combinatorial mixing disc are as follows.

- A disc design for 6x4 combinatorial mixing was demonstrated
- Numerous fluidic tests demonstrated the successful metering of all components on disc
- The integration of one whole sheet of DF was utilised to reliably place 96 DF tabs
- The multi-layer architecture permitted complex liquid transportation around the entirety of the disc
- Novel valving system demonstrated a time delay sequence which permitted successful metering around the entirety of the disc
- Generation of twenty-four unique sample/reagent outputs

7.1.3 10x10 Combinatorial Mixing Disc

The outcomes of the 10x10 combinatorial mixing disc are as follows.

- A disc design for 10x10 combinatorial mixing was demonstrated
- The integration of two whole sheets of DF were used to reliably place two hundred DF tabs
- The novel valving system demonstrated the utilisation of two DF sheets to isolate two components
- Demonstrated the potential of a highly scalable microfluidic combinatorial mixing system which could potentially be miniaturised

7.2 Future Work

There are a number of different aspects surrounding this work which could be explored in more detail in the future.

7.2.1 Pathogen Testing

This work focused on pathogens, BOTY and TYLCV, relating to tomato crop devastation. The disc design and development could be used to test for a range of other pathogenic contaminants including *E.coli* (Shiga toxin one and Shiga toxin two). A wide variety of food products contaminated with *E.coli* have shown to exhibit unfavourable symptoms after human consumption and so could be detected using combinatorial mixing systems for on-site applications [104]. Carrying out testing for various pathogenic contaminants would show the versatile capabilities of these combinatorial mixing systems.

7.2.2 Thermal Heating Unit with Fluorescence Detector

Throughout the course of this work a standard laboratory oven was used to simulate the isothermal conditions required to perform LAMP. Although this worked well the overall process would benefit from the manufacture of a thermal heating unit integrated onto a test spin-stand. This would allow for immediate heating of the samples loaded onto the disc after microfluidic testing has been completed. This could be carried out using inexpensive materials such as spring-loaded thermal blocks or by a control-heated air gun. The integration of a fluorescence detection unit would also greatly improve the overall detection system as this would allow all analysis of samples to be carried out on the spin-stand in an autonomous manner. There have been various elements used for heating microfluidic chips in the literature to date which include thermal blocks and air guns and

have been previously explored throughout this thesis [105]. These approaches have generated the best results and so could be integrated easily into this detection system.

7.2.3 Design of Full Sample-to-Answer System

This work focused on the amplification of purified DNA with sample preparation carried out on bench in a microfluidic system. Another progression would see the combination of DNA extraction from a food sample, DNA purification and sample preparation on a single device. This is the ultimate goal for a full sample-to-answer system which creates a user-friendly device which could be deployed for on-site applications. Integrating the MxN combinatorial mixing design into a DNA extraction disc could produce a fully functional sample-to-answer system.

There are great advantages associated with increased combinatorial power. One of the greatest advantages is reducing the pipetting steps from 2xMxN to M+N pipetting steps. The exponential relationship between the number of loading steps and the number of assays created means that any increase in integration density on the disc results in a major increase in screening throughput. There are great challenges associated with large scale integration and in particular around manufacturing and sealing of chips. Furthermore, reduction of sample and reagents volumes increases the surface to volume ratio which can have a detrimental effect on bio-assays. Therefore, such miniaturisation often requires laborious and time consuming assay optimisation. A further challenge is the need to almost continuously monitor a large number of samples. Therefore, the detection instrumentation would require capability to make many sample readings per second.

Research Profile

Papers

1. Macdara Glynn, Daniel Kirby, **Danielle Chung**, David Kinahan, Gregor Kijanka, Jens Ducrée. *Centrifugo-magnetophoretic purification of CD4+ cells from whole blood toward future HIV/AIDS point-of-care applications,* doi:10.1177/2211068213504759

2. Macdara Glynn, David Kinahan, **Danielle Chung** and Jens Ducrée. *CD4 cell isolation from blood using finger-actuated on-chip magnetophoresis for rapid HIV/AIDS diagnostics,* 10.1109/MEMSYS.2014.6765624.

Conference Manuscripts

1. **Danielle Chung**, David Kinahan, Cor Schoen, Tanja Dreo and Jens Ducrée. *Highly Scalable Combinatorial Mixing of Samples with Target-Specific Primers for Rapid Pathogen Detection on a Centrifugal Platform.* [Accepted to MicroTAS for poster presentation].

2. David Kinahan, Gregory Schneider, Yanina Cesa, **Danielle Chung** and Jens Ducrée. *On-site methods within DECATHLON- towards point-of-use molecular analysis and next generation sequencing.* [RME accepted]

3. David Kinahan, Gregory Schneider, Yanina Cesa, **Danielle Chung** and Jens Ducrée. *Microfluidic platforms towards sample preparation, nucleic acid identification and next generation sequencing for on-site applications.* [7th International Symposium on Recent Advances in Food Analysis]

The authorship of the two journal papers listed is based on my contribution to a prior project which was not part of this MSc thesis. My contribution to Kinahan *et al.* (RME) and Kinahan *et al.* (RAFA) was the work included in this thesis pertaining to optimisation of LAMP amplification on disc.

The content of Chung *et al.* is based on Chapter 4 of this MSc thesis. I planned this work, conducted the experiments and wrote the abstract. The combinatorial mixing on disc concept was developed by both me and David Kinahan. I developed the ideas and design for the disc and David used his high level of expertise to draw the design on SolidWorks. The manufacture and assembly of the microfluidic discs were carried out by me. All

experimental work was conducted by me to obtain the results presented throughout the work of this thesis.

Bibliography

[1] E. Scallan, R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin, "Foodborne illness acquired in the United States-Major pathogens," *Emerg. Infect. Dis.*, vol. 17, no. 1, pp. 7–15, 2011.

[2] I. M. Hanssen, M. Lapidot, and B. P. H. J. Thomma, "Emerging viral diseases of tomato crops.," *Mol. Plant. Microbe. Interact.*, vol. 23, no. 5, pp. 539–48, 2010.

[3] R. V. Tauxe, "Emerging Foodborne Diseases: An Evolving Public Health Challenge," *Emerg. Infect. Dis.*, vol. 3, no. 4, pp. 425–434, 1997.

[4] V. H. Tournas, "Spoilage of vegetable crops by bacteria and fungi and related health hazards.," *Crit. Rev. Microbiol.*, vol. 31, no. 1, pp. 33–44, 2005.

[5] M. W. Johnson, E. S. Sylvester, N. C. Toscano, K. Kid, H. T. Reynolds, and E. T. Natwick, "Whiteflies cause problems for southern California growers," *Carlifornia Agric.*, no. October, pp. 24–26, 1982.

[6] B. Hasiów-Jaroszewska and N. Borodynko, "Detection of Pepino mosaic virus isolates from tomato by one-step reverse transcription loop-mediated isothermal amplification," *Arch. Virol.*, vol. 158, no. 10, pp. 2153–2156, 2013.

[7] M. Amin Almasi, "Development of Colorimetric Loop-Mediated Isothermal Amplification Assay for Rapid Detection of the Tomato Yellow Leaf Curl Virus," *J. Plant Pathol. Microbiol.*, vol. 04, no. 01, pp. 1–6, 2012.

[8] R. Lenarčič, D. Morisset, N. Mehle, and M. Ravnikar, “Fast real-time detection of Potato spindle tuber viroid by RT-LAMP,” *Plant Pathol.*, vol. 62, no. 5, pp. 1147–1156, 2013.

[9] J. Yasuhara-Bell, R. Kubota, D. M. Jenkins, and A. M. Alvarez, “Loop-mediated amplification of the ’Clavibacter michiganensis subsp. michiganensis micA gene is highly specific.,” *Phytopathology*, vol. 103, no. 12, pp. 1220–1226, 2013.

[10] J. Hill, S. Beriwal, I. Chandra, V. K. Paul, A. Kapil, T. Singh, R. M. Wadowsky, V. Singh, A. Goyal, T. Jahnukainen, J. R. Johnson, P. I. Tarr, and A. Vats, “Loop-mediated isothermal amplification assay for rapid detection of common strains of Escherichia coli,” *J. Clin. Microbiol.*, vol. 46, no. 8, pp. 2800–2804, 2008.

[11] Z. R. Hansen, B. J. Knaus, J. F. Tabima, C. M. Press, H. S. Judelson, N. J. Grünwald, and C. D. Smart, “Loop-mediated isothermal amplification (LAMP) for detection of the tomato and potato late blight pathogen, Phytophthora infestans,” *J Appl Microbiol*, 2016.

[12] J. a Tomlinson, M. J. Dickinson, and N. Boonham, “Rapid detection of Phytophthora ramorum and P. kernoviae by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device.,” *Phytopathology*, vol. 100, no. 2, pp. 143–9, 2010.

[13] B. Malorny, P. T. Tassios, P. Rodstrom, N. Cook, M. Wagner, and J. Hoorfar, “Standardization of diagnostic PCR for the detection of foodborne pathogens,” *Int. J. Food Microbiol.*, vol. 83, no. 1, pp. 39–48, 2003.

[14] W. K. J. G. Schochetman C.-Y. Ou, “Polymerase Chain Reaction,” *J. Infect. Dis.*, vol. 158, no. 3, p. 1154, 1985.

[15] S. J. Oh, B. H. Park, J. H. Jung, G. Choi, D. C. Lee, D. H. Kim, and T. S. Seo, “Centrifugal loop-mediated isothermal amplification microdevice for rapid, multiplex and colorimetric foodborne pathogen detection,” *Biosens. Bioelectron.*, vol. 75, pp. 293–300, 2016.

[16] L. Niessen, J. Luo, C. Denschlag, and R. F. Vogel, “The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants,” *Food Microbiol.*, vol. 36, no. 2, pp. 191–206, 2013.

[17] G. M. Whitesides, “The origins and the future of microfluidics.,” *Nature*, vol. 442, no. 7101, pp. 368–73, 2006.

[18] S. Neethirajan, I. Kobayashi, M. Nakajima, D. Wu, S. Nandagopal, and F. Lin, “Microfluidics for food, agriculture and biosystems industries,” *Lab Chip*, vol. 11, no. 9, pp. 1574–1586, 2011.

[19] C. Black and H. G. Welch, “for Disease,” no. January, pp. 3–11, 1997.

[20] C. Lee, C. Grasso, and M. F. Sharlow, “The essence of SNPs.,” *Bioinformatics (Oxford, England)*, vol. 18, no. 3. pp. 452–64, 2002.

[21] N. A. Baird, P. D. Etter, T. S. Atwood, M. C. Currey, A. L. Shiver, Z. A. Lewis, E. U. Selker, W. A. Cresko, and E. A. Johnson, “Rapid SNP discovery and genetic mapping using sequenced RAD markers,” *PLoS One*, vol. 3, no. 10, pp. 1–7, 2008.

[22] A. Lauri and P. O. Mariani, “Potentials and limitations of molecular diagnostic methods in food safety.,” *Genes Nutr.*, vol. 4, no. 1, pp. 1–12, 2009.

[23] Bill Sardi, "No Title," 2014. [Online]. Available: https://www.lewrockwell.com/2014/07/bill-sardi/the-human-dna-debate/.

[24] J. E. Polston and P. K. Anderson, "of 7Hitef Ly 4Ransmitted ' Eminiviruses in 4Omato in the 7Estern (Emisphere," *Plant Dis.*, vol. 81, no. 12, 1995.

[25] R. Naravaneni and K. Jamil, "Rapid detection of food-borne pathogens by using molecular techniques," *J. Med. Microbiol.*, vol. 54, no. 1, pp. 51–54, 2005.

[26] V. Velusamy, K. Arshak, O. Korostynska, K. Oliwa, and C. Adley, "An overview of foodborne pathogen detection: In the perspective of biosensors," *Biotechnology Advances*, vol. 28, no. 2. pp. 232–254, 2010.

[27] P. K. Mandal, A. K. Biswas, K. Choi, and U. K. Pal, "Methods for Rapid Detection of Foodborne Pathogens: An Overview," *American Journal of Food Technology*, vol. 6, no. 2. pp. 87–102, 2011.

[28] P. Mead, "Food-Related Illness and Death in the United States Reply to Dr. Hedberg," *Emerg. Infect. Dis.*, vol. 5, no. 6, pp. 841–842, 1999.

[29] N. T. Perna, G. Plunkett 3rd, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grotbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamousis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner, "Genome sequence of enterohaemorrhagic Escherichia coli O157:H7," *Nature*, vol. 409, no. 6819, pp. 529–533, 2001.

[30] S. M. H. Qadri and S. Kayali, "Enterohemorrhagic *Escherichia coli*," *Postgrad. Med.*, vol. 103, no. 2, pp. 179–187, 1998.

[31] R. L. Buchanan and M. P. Doyle, “Foodborne disease significance of Escherichia coli O157:H7 and other enterohemorrhagic E. coli,” *Food Technol.*, vol. 51, no. 10, pp. 69–76, 1997.

[32] A. Alfaro-Fernandez, M. D. C. Cordoba-Selles, M. Juarez, J. ??ngel Herrera-Vasquez, J. Angel Sanchez-Navarro, M. D. C. Cebrian, M. I. Font, and C. Jorda, “Occurrence and geographical distribution of the ‘torrado’ disease in Spain,” *J. Phytopathol.*, vol. 158, no. 7–8, pp. 457–469, 2010.

[33] B. Pico, M. J. Diez, and F. Nuez, “Viral diseases causing the greatest economic losses to the tomato crop. II. The tomato yellow leaf curl virus - A review,” *Sci. Hortic. (Amsterdam).*, vol. 67, no. 3–4, pp. 151–196, 1996.

[34] H. Czosnek, “Tomato Yellow Leaf Curl Virus.” pp. 138–145, 2008.

[35] H. J. Rosslenbroich and D. Stuebler, “Botrytis cinerea - History of chemical control and novel fungicides for its management,” *Crop Prot.*, vol. 19, no. 8–10, pp. 557–561, 2000.

[36] E. M. Soylu, Ş. Kurt, and S. Soylu, “In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea,” *International Journal of Food Microbiology*, vol. 143, no. 3. pp. 183–189, 2010.

[37] GardnersHq, “No Title.” [Online]. Available: http://www.gardenershq.com/Growing-Tomatoes.php.

[38] Clemson, “No Title,” 2015. [Online]. Available: http://www.clemson.edu/extension/hgic/pests/plant_pests/veg_fruit/hgic2217.htm

[39] Plant Health Progress, “No Title,” 2006. [Online]. Available: https://www.plantmanagementnetwork.org/pub/php/research/2006/botrytis/.

[40] J. Juusola and J. Ballantyne, “mRNA profiling for body fluid identification by multiplex quantitative RT-PCR,” *J. Forensic Sci.*, vol. 52, no. 6, pp. 1252–1262, 2007.

[41] D. Zubakov, M. Kokshoorn, A. Kloosterman, and M. Kayser, “New markers for old stains: Stable mRNA markers for blood and saliva identification from up to 16-year-old stains,” *Int. J. Legal Med.*, vol. 123, no. 1, pp. 71–74, 2009.

[42] D. Zubakov, A. W. M. Boersma, Y. Choi, P. F. Van Kuijk, E. A. C. Wiemer, and M. Kayser, “MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation,” *Int. J. Legal Med.*, vol. 124, no. 3, pp. 217–226, 2010.

[43] R. Ogden, “Unlocking the potential of genomic technologies for wildlife forensics,” *Mol. Ecol. Resour.*, vol. 11, no. SUPPL. 1, pp. 109–116, 2011.

[44] R. Ogden, N. Dawnay, and R. McEwing, “Wildlife DNA forensics - Bridging the gap between conservation genetics and law enforcement,” *Endanger. Species Res.*, vol. 9, no. 3, pp. 179–195, 2009.

[45] P. A. Morin, G. Luikart, and R. K. Wayne, “SNPs in ecology, evolution and conservation,” *Trends Ecol. Evol.*, vol. 19, no. 4, pp. 208–216, 2004.

[46] D. J. Cahill, “Protein and antibody arrays and their medical applications,” *Journal of Immunological Methods*, vol. 250, no. 1–2. pp. 81–91, 2001.

[47] J. T. Auman and H. L. McLeod, “Cancer pharmacogenomics: DNA genotyping and gene expression profiling to identify molecular determinants of chemosensitivity.,” *Drug Metab. Rev.*, vol. 40, no. 2, pp. 303–315, 2008.

[48] P. J. Jannetto, E. Laleli-Sahin, and S. H. Wong, “Pharmacogenomic genotyping methodologies,” *Clin. Chem. Lab. Med.*, vol. 42, no. 11, pp. 1256–1264, 2004.

[49] A. K. Bej, M. H. Mahbubani, and R. M. Atlas, “Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications,” *Crit Rev.Biochem.Mol.Biol.*, vol. 26, no. 1040–9238 SB - IM, pp. 301–334, 1991.

[50] E. M. Elnifro, A. M. Ashshi, R. J. Cooper, and P. E. Klapper, “Multiplex PCR : Optimization and Application in Diagnostic Virology Multiplex PCR : Optimization and Application in Diagnostic Virology,” *Clin. Microbiol. Rev.*, vol. 13, no. 4, pp. 559–570, 2000.

[51] S. Yang and R. E. Rothman, “PCR-based diagnostics for infectious diseases: Uses, limitations, and future applications in acute-care settings,” *Lancet Infect. Dis.*, vol. 4, no. 6, pp. 337–348, 2004.

[52] R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, G. T. Horn, K. B. Mullis, H. A. Erlich, R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich, “Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1700278 REFERENCES Linked references are available on JSTOR for t,” vol. 239, no. 4839, pp. 487–491, 2016.

[53] "CGDP - DNA Amplification." [Online]. Available: https://www.flmnh.ufl.edu/cowries/amplify.html.

[54] D. Cai, M. Xiao, P. Xu, Y. Xu, and W. Du, "An Integrated Microfluidic Device Utilizing Dielectrophoresis and Multiplex Array PCR for Point-of-Care Detection of Pathogens," *Lab Chip*, vol. 14, no. 20, pp. 3917–3924, 2014.

[55] X. Zhao, C.-W. Lin, J. Wang, and D. H. Oh, "Advances in rapid detection methods for foodborne pathogens.," *J. Microbiol. Biotechnol.*, vol. 24, no. 3, pp. 297–312, 2014.

[56] K. E. Vrana, W. M. Freeman, H. D. Vanguilder, K. E. Vrana, and W. M. Freeman, "Twenty-five years of quantitative PCR for gene expression analysis," vol. 44, no. July 2016, pp. 619–626, 2008.

[57] J. R. Epstein, I. Biran, and D. R. Walt, *Fluorescence-based nucleic acid detection and microarrays*, vol. 469, no. 1. 2002.

[58] S. Tyagi and F. R. Kramer, "Molecular Beacons: Probes that Fluoresce upon Hybridization," *Nat. Biotechnol.*, vol. 14, no. 3, pp. 303–308, 1996.

[59] H. Zipper, H. Brunner, J. Bernhagen, and F. Vitzthum, "Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications," *Nucleic Acids Res.*, vol. 32, no. 12, 2004.

[60] L. A. Haft, "Improved Quantitative PCR Using Nested Primers," pp. 332–337, 1994.

[61] Y. Mori and T. Notomi, "Loop-mediated isothermal amplification (LAMP): A rapid, accurate, and cost-effective diagnostic method for infectious diseases," *J. Infect. Chemother.*, vol. 15, no. 2, pp. 62–69, 2009.

[62] W. Su, X. Gao, L. Jiang, and J. Qin, “Microfluidic platform towards point-of-care diagnostics in infectious diseases,” *J. Chromatogr. A*, vol. 1377, pp. 13–26, 2015.

[63] P. Gong, T. Zhang, F. Chen, L. Wang, S. Jin, and X. Bai, “Advances in loop-mediated isothermal amplification: integrated with several point-of-care diagnostic methods,” *Anal. Methods*, vol. 6, no. 19, pp. 7585–7589, 2014.

[64] D. S. Kryndushkin, I. M. Alexandrov, M. D. Ter-Avanesyan, and V. V. Kushnirov, “Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104.,” *J. Biol. Chem.*, vol. 278, no. 49, pp. 49636–49643, 2003.

[65] K. D. Reed, M. E. Stemper, and S. K. Shukla, “Pulsed-field gel electrophoresis of MRSA.,” *Methods Mol. Biol.*, vol. 391, no. 1, pp. 59–69, 2007.

[66] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase, “Loop-mediated isothermal amplification of DNA,” *Nucleic Acids Res*, vol. 28, no. 12, p. E63, 2000.

[67] D. Mabey, R. W. Peeling, A. Ustianowski, and M. D. Perkins, “Diagnostics for the developing world.,” *Nat. Rev. Microbiol.*, vol. 2, no. 3, pp. 231–240, 2004.

[68] E. L. A. Howson, B. Armson, M. Madi, C. J. Kasanga, S. Kandusi, R. Sallu, E. Chepkwony, A. Siddle, P. Martin, J. Wood, V. Mioulet, D. P. King, T. Lembo, S. Cleaveland, and V. L. Fowler, “Evaluation of Two Lyophilized Molecular Assays to Rapidly Detect Foot-and-Mouth Disease Virus Directly from Clinical Samples in Field Settings.,” *Transbound. Emerg. Dis.*, pp. 1–11, 2015.

[69] B. Deiman, P. van Aarle, and P. Sillekens, “Characteristics and applications of nucleic acid sequence-based amplification (NASBA).,” *Mol. Biotechnol.*, vol. 20, no. 2, pp. 163–179, 2002.

[70] T. Thorsen et al., “Microfluidic Large-Scale Integration,” *Sci. Mag*, vol. 298, no. 5593, pp. 580–4, 2002.

[71] R. Gorkin, J. Park, J. Siegrist, M. Amasia, B. S. Lee, J.-M. Park, J. Kim, H. Kim, M. Madou, and Y.-K. Cho, “Centrifugal microfluidics for biomedical applications,” *Lab Chip*, vol. 10, no. 14, pp. 1758–1773, 2010.

[72] S. O. Sundberg, C. T. Wittwer, C. Gao, and B. K. Gale, “Spinning disk platform for microfluidic digital polymerase chain reaction,” *Anal. Chem.*, vol. 82, no. 4, pp. 1546–1550, 2010.

[73] L. Marle and G. M. Greenway, “Microfluidic devices for environmental monitoring,” *TrAC - Trends Anal. Chem.*, vol. 24, no. 9, pp. 795–802, 2005.

[74] M. Amasia, M. Cozzens, and M. J. Madou, “Centrifugal microfluidic platform for rapid PCR amplification using integrated thermoelectric heating and ice-valving,” *Sensors and Actuators, B: Chemical*, vol. 161, no. 1. pp. 1191–1197, 2012.

[75] M. Focke, F. Stumpf, B. Faltin, P. Reith, D. Bamarni, S. Wadle, C. Müller, H. Reinecke, J. Schrenzel, P. Francois, D. Mark, G. Roth, R. Zengerle, and F. von Stetten, “Microstructuring of polymer films for sensitive genotyping by real-time PCR on a centrifugal microfluidic platform.,” *Lab Chip*, vol. 10, no. 19, pp. 2519–26, 2010.

[76] A. W. and D. D. B. Than Linh Quyen, Sun Yi, Wei Hoe Chin, Tran Quang Hung, Sannah Jardenbæk, “Eight-chamber microfluidic device with integrated loop mediated isothernmal amplification (LAMP) for multiple detection of Campylobacter spp from pig at slaughter,” *Proc. The18th Int. Conf. Miniaturized Syst. Chem. Life Sci.*, pp. 1930 – 1932, 2014.

[77] D. M. Tourlousse, F. Ahmad, R. D. Stedtfeld, G. Seyrig, J. M. Tiedje, and S. a Hashsham, “A polymer microfluidic chip for quantitative detection of multiple water- and foodborne pathogens using real-time fluorogenic loop-mediated isothermal amplification.,” *Biomed. Microdevices*, vol. 14, no. 4, pp. 769–78, 2012.

[78] D. R. Tobergte and S. Curtis, “Microfluidics- a review,” *J. Chem. Inf. Model.*, vol. 53, no. 9, pp. 1689–1699, 2013.

[79] L. X. Kong, A. Perebikovsky, J. Moebius, L. Kulinsky, and M. Madou, “Lab-on-a-CD: A Fully Integrated Molecular Diagnostic System.,” *J. Lab. Autom.*, p. 2211068215588456–, 2015.

[80] R. Burger, D. Kirby, M. Glynn, C. Nwankire, M. O’Sullivan, J. Siegrist, D. Kinahan, G. Aguirre, G. Kijanka, R. A. Gorkin, and J. Ducree, “Centrifugal microfluidics for cell analysis,” *Curr. Opin. Chem. Biol.*, vol. 16, no. 3–4, pp. 409–414, 2012.

[81] J. Ducrée, S. Haeberle, S. Lutz, S. Pausch, F. Von Stetten, and R. Zengerle, “The centrifugal microfluidic Bio-Disk platform,” *J. Micromechanics Microengineering*, vol. 17, no. 7, pp. S103–S115, 2007.

[82] R. Gorkin, C. E. Nwankire, J. Gaughran, X. Zhang, G. G. Donohoe, M. Rook, R. O'Kennedy, and J. Ducrée, "Centrifugo-pneumatic valving utilizing dissolvable films.," *Lab Chip*, vol. 12, no. 16, pp. 2894–902, Aug. 2012.

[83] G. S. Fiorini and D. T. Chiu, "Disposable microfluidic devices: Fabrication, function, and application," *Biotechniques*, vol. 38, no. 3, pp. 429–446, 2005.

[84] K. W. Oh and C. H. Ahn, "A review of microvalves," *J. Micromechanics Microengineering*, vol. 16, no. 5, pp. R13–R39, 2006.

[85] J. Siegrist, R. Gorkin, L. Clime, E. Roy, R. Peytavi, H. Kido, M. Bergeron, T. Veres, and M. Madou, "Serial siphon valving for centrifugal microfluidic platforms," *Microfluid. Nanofluidics*, vol. 9, no. 1, pp. 55–63, 2010.

[86] G. R., N. C.E., G. J., Z. X., D. G.G., R. M., O. R., and D. J., "Centrifugo-pneumatic valving utilizing dissolvable films," *Lab a Chip - Miniaturisation Chem. Biol.*, vol. 12, no. 16, pp. 2894–2902, 2012.

[87] N. Dimov, E. Clancy, J. Gaughran, D. Boyle, D. Mc Auley, M. T. Glynn, R. M. Dwyer, H. Coughlan, T. Barry, L. M. Barrett, T. J. Smith, and J. Ducr??e, "Solvent-selective routing for centrifugally automated solid-phase purification of RNA," *Microfluid. Nanofluidics*, vol. 18, no. 5–6, pp. 859–871, 2015.

[88] D. J. Kinahan, S. M. Kearney, N. Dimov, M. T. Glynn, and J. Ducrée, "Event-triggered logical flow control for comprehensive process integration of multi-step assays on centrifugal microfluidic platforms," *Lab Chip*, vol. 14, no. 13, pp. 2249–2258, 2014.

[89] W. Zhang, S. Lin, C. Wang, J. Hu, C. Li, Z. Zhuang, Y. Zhou, R. A. Mathies, and C. J. Yang, “PMMA/PDMS valves and pumps for disposable microfluidics.,” *Lab Chip*, vol. 9, no. 21, pp. 3088–94, 2009.

[90] Y. Chen, L. Zhang, and G. Chen, “Fabrication, modification, and application of poly(methyl methacrylate) microfluidic chips,” *Electrophoresis*, vol. 29, no. 9, pp. 1801–1814, 2008.

[91] H. Becker and L. E. Locascio, “Polymer microfluidic devices,” *Talanta*, vol. 56, no. 2, pp. 267–287, 2002.

[92] 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 Actuators, B Chem.*, vol. 99, no. 1, pp. 186–196, 2004.

[93] E. Berthier, E. W. K. Young, and D. Beebe, “Engineers are from PDMS-land, Biologists are from Polystyrenia,” *Lab Chip*, vol. 12, no. 7, p. 1224, 2012.

[94] A. Jagota and S. J. Bennison, “Mechanics of adhesion through a fibrillar microstructure.,” *Integr. Comp. Biol.*, vol. 42, no. 6, pp. 1140–5, 2002.

[95] P. Patil and S. K. Shrivastava, “Fast Dissolving Oral Films: An Innovative Drug Delivery System,” *Int. J. Sci. Res. ISSN (Online Impact Factor*, vol. 3, no. 7, pp. 2319–7064, 2012.

[96] M. F. Jensen, M. Noerholm, L. H. Christensen, and O. Geschke, “Microstructure fabrication with a CO2 laser system: characterization and fabrication of cavities produced by raster scanning of the laser beam.,” *Lab Chip*, vol. 3, no. 4, pp. 302–307, 2003.

[97] N. C. Nayak, Y. C. Lam, C. Y. Yue, and A. T. Sinha, “CO 2 -laser micromachining of PMMA: the effect of polymer molecular weight,” *J. Micromechanics Microengineering*, vol. 18, no. 9, p. 095020, 2008.

[98] D. A. Bartholomeusz, R. W. Boutté, and J. D. Andrade, “Xurography: Rapid prototyping of microstructures using a cutting plotter,” *J. Microelectromechanical Syst.*, vol. 14, no. 6, pp. 1364–1374, 2005.

[99] D. J. Kinahan, S. M. Kearney, N. Dimov, M. T. Glynn, and J. Ducrée, “Event-triggered logical flow control for comprehensive process integration of multi-step assays on centrifugal microfluidic platforms.,” *Lab Chip*, vol. 14, no. 13, pp. 2249–58, Jul. 2014.

[100] G. Zhang, E. W. Brown, and N. González-Escalona, “Comparison of real-time PCR, reverse transcriptase real-time PCR, loop-mediated isothermal amplification, and the FDA conventional microbiological method for the detection of Salmonella spp. in produce,” *Appl. Environ. Microbiol.*, vol. 77, no. 18, pp. 6495–6501, 2011.

[101] J. Melorose, R. Perroy, and S. Careas, “No Title No Title,” *Statewide Agricultural Land Use Baseline 2015*, 2015. [Online]. Available: https://en.wikipedia.org/wiki/Agarose_gel_electrophoresis.

[102] C. A. Kreader, “Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein,” *Appl. Environ. Microbiol.*, vol. 62, no. 3, pp. 1102–1106, 1996.

[103] N. McArdle, “Development of Large Scale Combinatorial Mixing on a Centrifugal Platform towards Genotyping Applications,” Dublin City University, 2016.

[104] M. Bielaszewska, R. Köck, A. W. Friedrich, C. von Eiff, L. B. Zimmerhackl, H. Karch, and A. Mellmann, “Shiga toxin-mediated hemolytic uremic syndrome: time to change the diagnostic paradigm,” *PLoS One*, vol. 2, no. 10, p. e1024, Jan. 2007.

[105] O. Strohmeier, M. Keller, F. Schwemmer, S. Zehnle, D. Mark, F. von Stetten, R. Zengerle, and N. Paust, “Centrifugal microfluidic platforms: advanced unit operations and applications.,” *Chem. Soc. Rev.*, vol. 44, pp. 6187–6229, 2015.